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PRINCIPAL INVESTIGATOR: Harry Joe, Ph.D.

CONTRACTING ORGANIZATION: The University of British Columbia
Vancouver, British Columbia, Canada V6T 1Z3

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6. AUTHOR(S)
Harry Joe, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of British Columbia
Vancouver, British Columbia, Canada V6T 1Z3

E-MAIL:
harry@stat.ubc.ca

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The large phenotypic variability of NF1 and NF2 complicates clinical management, confounds analysis of clinical treatment trials, limits the prognostic value of genetic counselling and adds substantially to the burden of the disease. The goals of this project are to devise new statistical methods to find patterns and relationships within the phenotypes and genotypes of people with NF, and to effectively model tumour formation in these disorders. In this way, we hope to be able to provide methods to better predict phenotype, enhance the effectiveness of genetic counselling and clinical screening, and aid in analysis of clinical trials' results.

This project describes research in statistical methods that would be useful for statistical modelling and analysis of clinical data from NF1 and NF2 subjects. The statistical methods are classified into the areas:
(a) estimation of familial correlation for different types of data,
(b) assessment of multi-hit mutation models for incidence of tumours.

Many of the statistical methods to be developed are either new or partly new and require further research for computer software implementation.

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Introduction

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This project describes research in statistical methods that would be useful for statistical modelling and analysis of clinical data from NF1 and NF2 subjects. The statistical methods are classified into the areas:
(a) estimation of familial correlation for different types of data,
(b) assessment of multi-hit mutation models for incidence of tumours.

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Clinical data exist in many formats including binary, categorical, count, and continuous information. Furthermore, a common "real life" problem is censored data (where the beginning or end point is not known for all cases but some intermediate data exist). . One goal of the project is to produce a software package for familial data analysis for different types of data, such as binary, count, and censored survival data.

Body

Purpose of the project

(A) To develop statistical methods that can be used to characterize the phenotype of individuals with NF1 and NF2.

(B) To develop methods to elaborate on the standard two-hit model of tumour formation, taking into account additional pathogenic factors and allelic differences for tumours in NF1 and NF2.

Research Accomplishments

The research accomplishments associated with each objective from the statement of work are summarized below.

Objective 1. *Develop statistical methods for interval-censored data, and obtain estimates of age of onset distributions for NF1 and NF2 features, using longitudinal information in the databases.*

This objective is being postponed as we currently do not have enough longitudinal information in the databases.

Objective 2. *Develop statistical methods for familial correlations for non-continuous and censored data, and obtain estimates of intraclass and interclass correlations for quantitative and binary traits in NF1 and NF2.*

Most of the progress in the past year, in the accepted papers and continuing PhD thesis work, has been in this objective. A summary of the statistical aspects of relevant accepted papers is given, followed by a report of newer statistical methods that are being developed for future use when the databases are larger.

In Baser et al AJHG (2002), a study was done of risk factors for mortality in people with neurofibromatosis 2 (NF2); this research also formed part of the MSc thesis of Dana Aeschliman (2002). Both clinical and molecular risk predictors were considered. If there were not observations from several members of each family, standard methods of survival analysis such as the Cox proportional hazards and log-normal survival regression models (Lawless, 1982) could be used. Because of the familial dependence for the NF2 families, we extend the log-normal survival model to a multivariate log-normal survival regression model from which intra-familial correlations can be estimated. Zhao, as part of her PhD thesis, is working on statistical theory that would allow intra-familial correlations or familial aggregation to be estimated when the Cox proportional hazards model is used (see below for more details).

For this paper, Aeschliman wrote a computer program for the estimation of familial correlations for right-censored survival data. A "new" statistical method in this paper is the use of the jackknife (Mosteller and Tukey, 1977) for familial data. It was used to estimate the standard errors of the regression parameters in Cox regression; the standard errors outputted from standard statistical software are too small because they don't take into account the positive association of measurements within families.

Another MSc student is improving the computer program of Aeschliman to estimate more general familial correlations for quantitative traits that are right censored. The improvement will make more use of the familial relationships. This module will be added to our software package when completed (see objective 4).

In Zhao et al (2002), the multivariate normal and log-normal survival regression models were used also for the response variables, age at onset of hearing loss and age at first symptom of NF2 for people with NF2, in order to estimate intra-familial correlations for different mutation types. The age at onset of hearing loss is right-censored because not all people with NF2 had developed hearing loss at their last clinical observation.

The other new development in this paper is a negative-binomial gamma mixture model to determine intrafamilial correlations of a count variable, such as number of intracranial meningiomas in NF2 non-probands. The variable, number of intracranial meningiomas, has a heavily right-skewed distribution and a sizeable frequency at zero, so that it was necessary to use a count distribution such as negative-binomial that can account for a large variance to mean ratio.

For further work in the next year, an MSc student will try to do a similar analysis with the count variable, number of spinal tumors, for NF2 patients. We will also consider the multivariate Poisson-log normal model of Aitchison and Ho (1989), in addition to the negative-binomial gamma mixture model.

Next we describe new developments in statistical methodology. Zhao, in continuing PhD research work, is studying theory for various estimating equation approaches that should lead to more reasonable computing time of familial associations for traits of the form of right-censored survival or count data, etc. An example of a count variable is the number of tumors of a particular type; an example of a right-censored survival variable is the age of onset of a particular disease feature - those who do not have the feature are right-censored at the age of last clinical observation.

This theory can be used for familial data, taking into account the relationships within families. Previous analysis in the above-mentioned paper used a simple exchangeable dependence model for familial dependence partly because the small sample size does not permit an analysis of whether intra-familial correlation decreases as the relation becomes more separated.

In Szudek et al (2002), more detailed intra-familial correlations for different classes of relations were estimated for various binary variables for presence/absence of features (e.g., Lisch nodules,

café-au-lait spots, subcutaneous neurofibromas, cutaneous neurofibromas, plexiform neurofibromas, intertriginous freckling). The genetic implications of the findings on intra-familial correlations are summarized in the abstract of this paper (please see the appendix).

In the future for the NF1, NF2, and other genetic diseases, it would be expected that more quantitative and longitudinal data would be recorded, such as the age at onset of features. Either count (eg. number of tumors) or quantitative variables (onset times) will provide more statistical power to compare intra-familial correlations than binary variables for presence/absence of disease features.

For most of the multivariate models for familial data, such as those with a latent multivariate normal distribution, the maximum likelihood approach is not computationally tractable when high-dimensional numerical integration is involved. It is desirable to develop other estimating approaches which are computationally less demanding, and relatively efficient (in the sense of variance of the sampling distribution of the estimator; see Rao, 1980). Methods based on composite likelihood (Lindsay, 1988) have been applied to other areas of multivariate data analysis, such as spatial data analysis (Lele, 1998), and multivariate survival data analysis (Parner, 2001). These approaches are being adapted for familial data. Mainly two types of composite likelihood are considered.

1. Univariate composite likelihood (UCL): composite likelihood formed by adding together the log-likelihood of univariate margins for each individual; this does not make use of the information on familial dependence.
2. Bivariate composite likelihood (BCL): composite likelihood formed by adding together the log likelihoods of bivariate margins from pairs of related individuals.

Estimation from composite likelihoods is equivalent to estimation via estimating equations (Godambe, 1991), with the estimating equations consisting of the vector of derivatives of the composite likelihood with respect to the model parameters. Classical maximum likelihood estimation is fine for continuous or quantitative responses (assumed transformed to the normal distribution), and for binary responses if the family sizes are ≤ 8. For other cases such as survival data with right-censored observation, or count data or binary responses with large family sizes, maximum likelihood estimation with standard multivariate familial models would require very-time consuming multidimensional numerical integration, so this leads to our consideration of other estimation methods such as composite likelihood methods. A standard comparison of efficiency of different estimation methods (asymptotic variance of parameter estimates) is via the Godambe information matrix.

From the above-mentioned UCL and BCL, the focus is on two estimating approaches. The first is a two-stage approach. In step 1, parameters which are identifiable by the univariate marginal distribution (marginal parameters) are estimated by maximizing the UCL function. In step 2, the dependence parameters are estimated by the BCL function with the marginal parameters replaced by their estimates from the first step.

It has been pointed out that the performance of the estimators can be pooled under certain circumstances (Lindsey, 1988). Different weighting schemes were also considered to enhance the efficiency of this approach. The second approach is based solely on BCL. The parameters are estimated simultaneously by maximizing the BCL function. To improve the efficiency, the contribution of each family is weighted by the family size.

The estimates based on composite likelihood are asymptotically consistent and unbiased. The estimates are much easier to compute under many circumstances compared to the maximum likelihood estimates. The major concern of these approaches is the efficiency. Comparisons of the relative efficiency of these two approaches against the maximum likelihood method are being made. Different models, including multivariate normal, multivariate probit, lognormal-Poisson mixture and multivariate lognormal with right censoring, have been examined analytically or by Monte Carlo simulation. In these models, there are three types of parameters: regression or covariate coefficients, dispersion parameters and dependence parameters.

Major findings to date are briefly summarized in the following:

(a) In a generalized regression model, efficiency of the estimate of a covariate coefficient generated from the UCL decreases when the correlation within families increases. When the intra-familial correlation is 1, the efficiency can decrease to zero for some models. By using weights or the BCL approach, the efficiency is much improved. Even when the correlation is moderately high, the efficiency is close to 1.

(b) In both approaches, the performance of the dispersion and dependence parameter estimates is generally satisfactory. In most cases, the efficiency is above 0.8. In comparison to the two-stage approach, the BCL approach performs better when the association is strong, but is less efficient when the association is weak.

(c) For both approaches, the efficiency depends on the composition of families. In general, when there are small portion of large families mixed with small families, the composite likelihood approaches suffer more efficiency loss than when the family size only varies a little.

Another direction of research is to use the Cox proportional hazard (PH) model (Cox, 1972) for multivariate survival data, with estimates of intra-familial correlations. Bandeen-Roche and Liang (1996) proposed a model for clustered data in which the univariate margin is formulated by the Cox model, and the joint distribution is specified by a one-parameter copula. Their model only allows exchangeable dependence structure. For our application to familial data, the multivariate normal distribution or copula will allow us to include more general dependence structures to cover a variety of family relations.

Objective 3. *Fit multi-hit mutation models for the incidence of NF2 and NF1 tumours by age, distinguish whether a two-hit or three-hit model provides a better fit to the data, and adapt the models to account for mutation type and other factors.*

Two- and three-hit models for vestibular schwanomas were fit to data for NF2 subjects; this was the MSc thesis work of Woods. Since then, some modifications have been done with different values that are relevant to the growth of Schwann cells. These results appear in Woods et al. (submitted manuscript, 2002) Genotype-phenotype correlations have been reported in subjects with NF2 and a model that incorporates a subject's genotype has been fit. With the latest NF2 database, which has more data on mutation type, we have compared the two-hit and three-hit models for vestibular schwanomas, but the conclusions regarding possible genotype-phenotype correlations do not appear strong. We may need to wait for a larger database to do a better analysis.

Objective 4. *Write C code to implement all of these statistical methods and provide a user-friendly interface for the code.*

Software written in C/C++, is being developed in Unix/Linux; it runs also in Windows with Cygnus/Gnuwin [see www.cygwin.com], the public domain version of Unix for Windows. The implementation of the interface currently is through a control file which specifies parameters and data files. By the end of 2001, methods for binary and quantitative (continuous) traits had been integrated into a computer package and were used in the statistical analysis in Szudek et al (2002).

This package has also been used more recently for analysis of the binary variable, presence/absence of cataract in NF2 patients. ["Are there genotype-phenotype correlations for presenile cataracts in neurofibromatosis 2?" Research manuscript in draft stage]

The next addition, with estimated completion time being December 2002, is a module for the handling of familial survival data based on multivariate normal distribution.

New modules based Zhao's PhD thesis research will be added after the theoretical work is further along.

The latest version of the software package can be obtained from the directory ftp://ftp.stat.ubc.ca/pub/hjoe/famil/

Summary

The summary according to the proposed timeline of work is given below.

0-12 months :
- development of statistical theory for the simpler cases,
- coding into C programs and use on current NF1/NF2 databases

12-24 months :
- extension of theory to cover more general situations
- continuation of coding and data analysis
- presentation of preliminary results in technical papers and conferences

The theory for simplest cases has been mostly developed for objectives 2 and 3. The coding into C programs and use on current NF1/NF2 databases has been completed for some of the methods. Four manuscripts have been accepted for publication, and 2 others have been submitted. Some presentations were made at the 2000 and 2001 meetings of the American Society of Human Genetics.

24-36 months :
- writing more general publications,
- conversion of C code to a form with friendlier input instructions, so that a non-computer programmer can use the computer programs.

Key Research Accomplishments

- Comparison of two- and three-hit models for onset time of vestibular schwanomas for NF2 subjects.

- Start of software package for analysis of familial data of various types (binary, count, continuous, censored). The software written in the C/C++ programming languages, developed in Unix/Linux, runs also in Windows with Cygnus/Gnuwin (public domain version of Unix for Windows).

- Application of the software package for estimating familial associations for NF1 and NF2 clinical features, with adjustments for the age effect.

- Development of statistical methodology that will be used to analyze NF databases in the future when there are more quantitative and longitudinal information.

Reportable Outcomes

(Please see appendix for listing of submitted papers and abstracts)

Completed Thesis Project

Dana Aeschliman (2001). Survival Times of NF2 Patients. September 2001.

Papers Accepted for Publication

Baser ME, Friedman JM, Aeschliman D, Joe H, Wallace AJ, Ramsden RT, Evans DGR (2002). Predictors of the risk of mortality in neurofibromatosis 2. (*Am J Hum Genet*, accepted May 2002).

Baser ME, Friedman JM, Wallace AJ, Ramsden RT, Joe H, Evans DGR (2002). Evaluation of clinical diagnostic criteria for neurofibromatosis 2. (*Neurology*, accepted July 2002)

Szudek J, Joe H, and Friedman JM (2002). Analysis of intra-familial phenotypic variation in neurofibromatosis 1 (NF1). *Genet Epidemiol in press*

Zhao Y, Kumar RA, Baser ME, Evans DGR, Wallace A, Kluwe L, Mautner VF, Parry DM, Rouleau GA, Joe H, Friedman JM (2002). Intrafamilial correlation of clinical manifestations in neurofibromatosis 2 (NF2). (*Genet Epidemiol*, accepted July 2002).

Published Abstracts

Tzenova J, Joe H, Riccardi VM, Friedman JM. The effect of parental age on the occurrence of neurofibromatosis 1.
Am J Hum Genet 69 (Suppl):393, 2001.

Zhao Y, Kumar RA, Baser ME, Evans DGR, Wallace A, Rouleau GA, Mautner VF, Kluwe L, Joe H, Friedman JM. Allele class-independent intrafamilial correlation of age at onset, age at hearing loss and number of intracranial meningiomas in neurofibromatosis 2 (NF2). Am J Hum Genet 2001;69(4 Suppl):A420.

Accepted Abstracts

Baser ME, Friedman JM, Joe H, Wallace AJ, Ramsden RT, Evans DGR. Genotype-phenotype correlations for presenile cataracts in neurofibromatosis 2. American Society of Human Genetics 52nd Annual Meeting, 2002 October 15-19, Baltimore (MD). Accepted.

Scientific Poster Presentations at National or International Meetings

Tzenova J, Joe H, Riccardi VM, Friedman JM. The effect of parental age on the occurrence of neurofibromatosis 1. . American Society of Human Genetics 51st Annual Meeting, 2001.

Zhao Y, Kumar RA, Baser ME, Evans DGR, Wallace A, Rouleau GA, Mautner VF, Kluwe L, Joe H, Friedman JM. Allele class-independent intrafamilial correlation of age at onset, age at hearing loss and number of intracranial meningiomas in neurofibromatosis 2 (NF2). American Society of Human Genetics 51st Annual Meeting, 2001.

Conclusions

As discussed in the previous year's report, progress on objective 1 has been limited by data availability. We anticipate that this situation will be somewhat mitigated in the next year by the availability of more published data and also by the development of an NF1 and NF2 genotype-phenotype database in the Friedman Lab at UBC, in Vancouver. This will consist of a new source of data for both diseases.

The original goal of objective 2, the development of techniques to analyze familial correlations, has been mostly completed and has been extended into areas which were not originally proposed but which are within the logical scope of this statistical research.

In objective 3, we developed and tested multi-hit models for tumour development. The methods have been developed successfully for vestibular schwannomas and have since been adapted to model Schwann cell growth. Although the original goals for this objective have been completed insofar as the methods developed appear valid, we need further clinical data to extend this work.

The first goal of objective 4 consists of writing C code to implement the above statistical methods. This has been done for the existing methods. Additional methods will be added in the next year after development of further theory. The second goal is to make it readily accessible with a user-friendly interface. This will be completed in the final year of this grant.

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Appendix

Preprints of the articles and abstracts listed below follow in this appendix

Articles

Baser ME, Friedman JM, Aeschliman D, Joe H, Wallace AJ, Ramsden RT, Evans DGR (2002). Predictors of the risk of mortality in neurofibromatosis 2.
(*Am J Hum Genet*, accepted May 2002).

Baser ME, Friedman JM, Wallace AJ, Ramsden RT, Joe H, Evans DGR (2002). Evaluation of clinical diagnostic criteria for neurofibromatosis 2.
(*Neurology*, accepted July 2002)

Palmer V, Szudek J, Joe H, Riccardi VM, and Friedman JM (2002). Analysis of neurofibromatosis 1 (nf1) lesions by body segment
(*Am J Med Genet*, submitted for publication)

Szudek J, Joe H, and Friedman JM (2002). Analysis of intra-familial phenotypic variation in neurofibromatosis 1 (NF1). *Genet Epidemiol in press*

Woods R, Friedman JM, Evans DGR, Baser ME, and Joe H (2002). Exploring the `2-hit hypothesis' in nf2: tests of 2-hit and 3-hit models of vestibular schwannoma development.
(*Genet Epidemiol,* submitted for publication)

Zhao Y, Kumar RA, Baser ME, Evans DGR, Wallace A, Kluwe L, Mautner VF, Parry DM, Rouleau GA, Joe H, Friedman JM (2002). Intrafamilial correlation of clinical manifestations in neurofibromatosis 2 (NF2). (*Genet Epidemiol*, accepted July 2002).

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Baser ME, Friedman JM, Joe H, Wallace AJ, Ramsden RT, Evans DGR. Genotype-phenotype correlations for presenile cataracts in neurofibromatosis 2. American Society of Human Genetics 52nd Annual Meeting, 2002 October 15-19, Baltimore (MD). Accepted.

Tzenova J, Joe H, Riccardi VM, Friedman JM. The effect of parental age on the occurrence of neurofibromatosis 1. *Am J Hum Genet 69 (Suppl):393, 2001.*

Zhao Y, Kumar RA, Baser ME, Evans DGR, Wallace A, Rouleau GA, Mautner VF, Kluwe L, Joe H, Friedman JM. Allele class-independent intrafamilial correlation of age at onset, age at hearing loss and number of intracranial meningiomas in neurofibromatosis 2 (NF2). *Am J Hum Genet;*69(4 Suppl):255, 2001.

Predictors of mortality in neurofibromatosis 2

Michael E. Baser, Ph.D.,[1] J. M. Friedman, M.D., Ph.D.,[2]

Dana Aeschliman, M.S.,[3] Harry Joe, Ph.D.,[3]

Andrew J. Wallace, Ph.D.,[4] Richard T. Ramsden, M.D.,[5]

D. Gareth R. Evans, M.D.[4]

[1]Los Angeles, U.S.A., [2]Department of Medical Genetics, University of British Columbia, Vancouver, Canada, [3]Department of Biostatistics, University of British Columbia, Vancouver, Canada, [4]Department of Medical Genetics, St. Mary's Hospital, Manchester, U.K., [5]Department of Otolaryngology, Manchester Royal Infirmary, Manchester, U.K.,

Address correspondence to:

Michael E. Baser, Ph.D.

2257 Fox Hills Dr., Los Angeles, CA 90064

tel 310-203-0351, fax 310-472-3614, e-mail baser@earthlink.net

Presented in part at the 49th Annual Meeting of the American Society of Human Genetics, Am J Hum Genet 1999;65(4 Suppl):A61.

Abstract

To evaluate clinical and molecular predictors of the risk of mortality in people with neurofibromatosis 2 (NF2), we analyzed the mortality experience of 350 patients from 240 families in the United Kingdom NF2 registry using the Cox proportional hazards model and the jackknife method. Age at diagnosis, intracranial meningiomas, constitutional *NF2* missense mutations, and type of treatment center were independent predictors of the risk of mortality. In Cox models, the relative risk of mortality increased 3.32-fold per decade decrease in age at diagnosis (95% confidence interval [CI], 1.73 - 6.36; $P < .001$), and 2.32-fold in people with meningiomas (95% CI, 1.19 - 4.51; $P = .013$), compared to those without meningiomas. The relative risk of mortality in patients treated at specialty centers was 0.34 (95% CI, 0.12 - 1.02; $P = .054$), compared to those treated at non-specialty centers. The relative risk of mortality in people with missense mutations was 0.01 (95% CI, 0.10 - 0.38; $P = .012$), compared to those with nonsense or frameshift mutations. We conclude that age at diagnosis, the strongest single predictor of the risk of mortality, is a useful index for patient counseling and clinical management (as is the presence of meningiomas), and that NF2 patients should be referred to specialty treatment centers for optimal care.

Introduction

Neurofibromatosis 2 (NF2) is an autosomal dominant disorder that is caused by inactivating mutations or loss of the *NF2* tumor suppressor gene.[1,2] Vestibular schwannomas (VSs), intracranial meningiomas, spinal tumors, peripheral nerve tumors, and presenile lens opacities commonly occur in NF2.[3-6] VSs are found in about 90% of adult NF2 patients (bilateral VSs are pathognomonic for NF2), meningiomas in about 50%, spinal tumors in about 90%, and presenile lens opacities in about 60-80% of patients.

Cross-sectional studies of genotype-phenotype correlations in NF2 have found that constitutional nonsense and frameshift *NF2* mutations generally are associated with severe disease, missense mutations and large deletions with mild disease, and splice-site mutations with variable disease severity.[7-13] Due to the rarity of NF2,[14,15] there have been few long-term longitudinal studies of the disease. Evans et al.[3] found that mean actuarial survival was 62 years, and Parry et al.[4] reported that broad categories of NF2 disease severity (mild, intermediate, severe) were correlated with age at death. However, neither of these studies evaluated specific clinical or molecular characteristics as potential predictors of the risk of mortality.

VS growth rates in NF2 patients are highly variable, but tend to be higher in people with a younger age at onset or diagnosis of NF2.[16,17] Inherited cases comprise half of all NF2 patients,[3] and there is high variability in VS growth rates even among affected relatives of similar ages in a single family.[17] In addition to VSs, NF2-associated meningiomas, spinal tumors, and their sequelae cause considerable morbidity in NF2.[18] NF2 is a chronic disease in which life expectancy, though often shortened, is lengthy. For these reasons, short-term studies of NF2 tumor growth rates, especially studies of a single tumor type such as VSs, do not reflect the total disease burden or efficacy of treatment as well as long-term studies that utilize a more

inclusive measure of health, such as mortality. In this study, we evaluated specific clinical and molecular risk factors for mortality in a large NF2 patient series.

Methods

Patient population

The United Kingdom NF2 registry is based in the Department of Medical Genetics, St. Mary's Hospital, Manchester. Patients are ascertained by contacting neurosurgeons, otolaryngologists, neurologists, pediatricians, dermatologists, and geneticists throughout the United Kingdom, augmented in the North West Region by the Regional Cancer Registry. As of 1 August 2001, the registry included 400 people from 261 families. For this study, we excluded two groups of patients:

(1) Somatic mosaics (N = 16). Almost all reported *NF2* somatic mosaics have mild disease, despite having nonsense or frameshift mutations.[19,20]

(2) People who were born before 1930 (N = 34). All such individuals in the United Kingdom NF2 registry were identified through younger affected relatives. The pre-1930 group was excluded because it did not meet the proportional hazards assumption for the Cox analysis. Specifically, type of treatment center and meningiomas did not predict the risk of mortality in people who were born before 1930, in contrast to those born after 1930.

The resultant study group had 350 people from 240 families, all of whom met the Manchester clinical diagnostic criteria for NF2[3] or had identified constitutional *NF2* mutations. The distribution of family sizes was: 186 families with one person, 24 families with two people, 17 families with three people, five families with four people, five families with five people, one

family with six people, and two families with seven people. There were 209 founders and 141 inherited cases.

NF2 mutation analysis

Genomic DNA samples prepared from peripheral lymphocytes were amplified with primers for all 17 exons of the *NF2* gene, and screening for constitutional *NF2* mutations using single-strand conformation polymorphism (SSCP) analysis was performed as described previously.[11]

Statistical analysis

Potential predictors of the risk of mortality were first assessed using univariate Kaplan-Meier survival curves. The covariates examined were age at onset of symptoms, age at diagnosis, gender, type of constitutional *NF2* mutation, inheritance (founder or inherited case), presence and number of each type of NF2 nervous system tumor (VSs, intracranial meningiomas, spinal tumors, and peripheral nerve tumors), lens opacities, number of surgical operations, calendar year of diagnosis, and type of treatment center (specialty or non-specialty). The specialty treatment centers, defined as hospitals with NF2 specialist management teams, were Manchester Royal Infirmary, Addenbrooke's Hospital (Cambridge), and Royal London Hospital. Each of these hospitals also had at least 20 NF2 patients in the registry. John Radcliffe Infirmary (Oxford) is also a specialty treatment center, but has been so for only a few years, and NF2 patients are referred to Manchester.

The covariates that were significantly associated with the risk of mortality in univariate analyses were included in Cox proportional hazards models with fixed covariates. The number of tumors at diagnosis was used because data from serial examinations were not routinely available. Interactions between age at diagnosis and number of each type of nervous system

tumor were evaluated because the penetrance of nervous system tumors in NF2 increases with age.[21,22] Age at onset of symptoms and age at diagnosis were highly correlated ($r^2 = .64$, $P < .001$); age at diagnosis was used in the analysis because tumor burden was first evaluated at this time. Age at diagnosis and calendar year of diagnosis were not highly correlated ($r^2 = .00$, $P = .20$).

Separate Cox models were used for clinical and molecular features because constitutional *NF2* mutation type is strongly associated with age at diagnosis and intracranial meningiomas.[7-13] The covariates in the clinical model were age at diagnosis, meningiomas (different models for presence of meningiomas and number of meningiomas), type of treatment center, number of surgical operations, and calendar year of diagnosis. The covariates in the molecular model were type of constitutional *NF2* mutation (splice-site mutations, missense mutations, and large deletions, each compared to nonsense or frameshift mutations), as well as type of treatment center and calendar year of diagnosis, two clinical covariates that were potential confounders, not known to be associated with mutation type. The molecular model excluded 77 people who had not been screened for constitutional *NF2* mutations.

Initially, we examined Cox models based only on founders. Founder/non-founder and proband/non-proband status are highly correlated, and proband status was not an independent predictor of mortality. The Cox model assumes independence of families and independence of members within families. The latter part of this assumption is violated when there is correlation between family members. When the data are positively correlated, the standard errors of parameters estimated under the assumption of independence will tend to be too small, which could lead to an erroneous conclusion that an effect is important. To surmount this problem for the Cox models that included both founders and inherited cases, the jackknife method was used

to calculate the parameters and their standard errors in the Cox model. The jackknife is a standard statistical method that is commonly used to correct for bias in an estimate and to provide robust interval estimation.[23] The distributions of such estimators will deviate from the usual χ^2 distributions, but two omnibus statistics were jackknifed to make inferences about the utility of the entire model. One statistic was the difference in –2(log(likelihood)) when no coefficients (the null model) and then all coefficients (the complete model) are included in the model. The other statistic was the efficient score test.[24]

To assess the amount of intrafamilial correlation, we created a model in which the $\log_{10}$ of lifespan was the response and each family was allocated a random effect. This allows for differing intercepts across families. We then compared the estimated variance of the random effect for families against the estimated error variance to obtain an estimate of the intrafamilial correlation. Distinct families were assumed to be independent.

Results

The characteristics of the NF2 study population are presented in Table 1. The mean $\pm$ SE age at onset of symptoms was 22 $\pm$ 1 years and the mean age at diagnosis was 27 $\pm$ 1 years. The median length of follow-up from diagnosis was five years (range, 0-35 years). Ninety-seven per cent of people were diagnosed after 1971. Ninety-three per cent of people had VSs and 45% had intracranial meningiomas. Constitutional *NF2* mutations were identified in 105 of the 175 families (60%) that were screened for mutations. Seventy-four (21%) of the 350 patients died during follow-up: 53 from tumor burden, 12 post-operatively, three from malignancies arising from an NF2 tumor, two each from road traffic accidents and suicide, and one each from a fall due to NF2-associated imbalance and myocardial infarction.

In univariate analyses based on all patients (founders and inherited cases), five covariates were associated with the risk of mortality: age at diagnosis, intracranial meningiomas, type of constitutional *NF2* mutation, type of treatment center, and calendar year of diagnosis. Kaplan-Meier survival curves are presented in Figures 1 - 4. These covariates were included in the Cox models.

In the clinical model based only on founders, age at diagnosis and meningiomas were independent predictors of the risk of mortality. The relative risk of mortality increased 4.26-fold per decade decrease in age at diagnosis (95% confidence interval [CI], 3.11 - 5.84; $P < .001$), and 2.15-fold in people with meningiomas (95% CI, 1.32 - 3.50; $P = .002$), compared to those without meningiomas. In a separate model that included number of meningiomas instead of presence of meningiomas, the relative risk of mortality increased 1.09-fold per meningioma (95% CI, 1.00 - 1.19; $P = .039$). When type of treatment center was included in the model, the relative risk of mortality in people who were treated in specialty centers was 0.47 (95% CI, 0.19 - 1.56; $P = .10$), compared to those treated in non-specialty centers. In the molecular model based only on founders, none of the mutation types were significant predictors of the risk of mortality, although as expected, patients with splice-site mutations, missense mutations, and large deletions each had a lower risk of mortality than those with nonsense or frameshift mutations.

In the clinical model based on founders and inherited cases, age at diagnosis, presence of meningiomas, and type of treatment center were independent predictors of the risk of mortality (Table 2). The relative risk of mortality increased 3.32-fold per decade decrease in age at diagnosis (95% CI, 1.73 - 6.36; $P < .001$), and 2.32-fold in people with meningiomas (95% CI, 1.19 - 4.51; $P = .013$), compared to those without meningiomas. In a separate model with

number of meningiomas instead of presence of meningiomas, the relative risk of mortality increased 1.08-fold per meningioma (95% CI, 0.98 - 1.19; P = .139). The relative risk of mortality in patients treated at specialty centers was 0.34 (95% CI, 0.12 - 1.02; P = .054), compared to those treated at non-specialty centers. Age was the strongest predictor, by far, of mortality, while meningiomas and type of treatment center were of similar quantitative importance. The clinical model accounted for 54% of the variance in risk of mortality.

In the molecular model based on founders and inherited cases, *NF2* missense mutations were an independent predictor of the risk of mortality (Table 2). The relative risk of mortality in people with missense mutations was 0.01 (95% CI, 0.10 - 0.38; P = .012), compared to those with nonsense or frameshift mutations. The discrepancy between the Cox model based only on founders and the model based on both founders and inherited cases is due to the fact that people with missense mutations generally have mild disease, have relatively large families, and as a result, there are few founders. There were 22 people with missense mutations in this study, but only five were founders.

In the entire group of 350 people, there may be a positive correlation of length of survival among family members, but the correlation is difficult to quantify with certainty due to the relatively small amount of data and large amount of censoring. The point estimate of the intra-familial correlation is 0.35 (95% CI, 0.06 - 0.50), and was calculated as follows. Separate models were constructed for age at diagnosis, presence of meningiomas, and type of treatment center. In each model, maximum likelihood estimation was used to estimate the regression coefficient, overall mean, error variance, and correlation between affected family members. The estimates of the regression coefficients and overall mean were fixed, and the values of the error variance and intra-familial correlation that maximize this restricted likelihood were found. This

method is inferior to simultaneous estimation of all parameters, but the method is dictated by the relatively small amount of data. There was also a strong intra-familial correlation of age at diagnosis, which possibly could be incorporated into the model if additional data become available.

Discussion

In this study, we found that four covariates were independent predictors of the risk of mortality in NF2. The risk of mortality was greater with decreasing age at diagnosis, and in people with meningiomas. The risk of mortality was lower in people with missense mutations relative to those with nonsense or frameshift mutations, and in people who were treated at specialty treatment centers relative to those who were treated in non-specialty centers. The simplicity of age at diagnosis, by far the strongest single predictor of risk of mortality in NF2, makes it a useful index for patient counseling and clinical management. The presence of meningiomas is also useful in this regard. A possible reason for higher risk of mortality in NF2 patients who are diagnosed at younger ages is the generally more rapid tumor growth in these patients,[16,17] perhaps due to higher rates of somatic cell growth or proportions of growing cells in young people. Age at onset (which is highly correlated with age at diagnosis) and number of non-VS intracranial tumors are key indices of NF2 disease severity.[4] Other covariates, such as gender, were not independent predictors of the risk of mortality.

Empirically, type of treatment center is of similar importance to intracranial meningiomas in the clinical model. In all likelihood, a major cause of the lower risk of mortality in NF2 patients who are treated in specialty centers is more extensive surgical experience and more favorable operative outcomes due to the larger number of surgeries in these centers. Buchman et

al.[25] found that, for postoperative preservation of facial nerve function in VS surgery, approximately 60 surgeries were needed before a new team achieved results similar to those of highly experience surgeons; there were also trends in improved complete resection rate and hearing preservation, and lowered incidence of cerebrospinal fluid leaks. In Denmark, decentralized VS neurosurgery was associated with very high rates of perioperative mortality (8.5%) and serious surgical complications (35.6%).[26] Patients with benign meningioma who are treated in academic hospitals have significantly lower mortality, after adjustment for other risk factors, than those who are treated in community hospitals.[27] In addition to more extensive surgical experience, specialty centers have coordinated expertise in the multiple clinical specialties that are needed to properly diagnose and treat NF2 patients and their at-risk family members.[28,29] We therefore recommend that NF2 patients be referred to specialty centers for optimal care.

Relatively few NF2 patients with access to treatment die from their VSs. In the modern era of improved microsurgical techniques, operative mortality in specialized neurotology treatment centers is 1% or lower, and recurrance rates are nil when the entire VS and vestibular nerves are excised. Intracranial meningiomas and spinal tumors are common in NF2, and these recurrent tumors often require repeated surgeries. There is considerable pre- and post-operative morbidity due to seizures, paralysis, wasting, pneumonia, and accidents associated with meningiomas and spinal tumors.[18]

Experimental studies have provided information on possible mechanisms through which constitutional *NF2* missense mutations cause mild disease relative to nonsense or frameshift *NF2* mutations. Gutmann et al.[30] demonstrated that missense mutations produced NF2 protein (termed merlin or schwannomin) that was stable but defective in negative growth regulation,

while nonsense mutations did not produce stable protein. Naturally-occurring missense mutations have reduced, but not absent, binding of the merlin-binding protein II-spectrin.[31]

Since *NF2* mutation type is strongly associated with age at diagnosis in cross-sectional studies, a logical question is why age at diagnosis is more strongly associated than mutation type with the risk of mortality. Age at diagnosis may have a stronger relationship to the risk of mortality because both age at diagnosis and age at death reflect a composite of disease-influencing factors, whereas mutation type is only one of these factors. Although NF2 disease severity is associated with type of constitutional *NF2* mutation in cross-sectional studies, other factors also play a role in determining phenotype. These factors include the stochastic loss of the second *NF2* allele[32] and genes other than *NF2* that may affect NF2 disease severity[33] and schwannoma[34,35] and meningioma[36] tumorigenesis.

Almost all of the people in this study were diagnosed after 1971. During the last three decades, there have been considerable improvements in neuroimaging techniques that have permitted earlier detection of small tumors. In combination with improvements in neurosurgical treatments, this has led to better clinical management and a much greater incentive to diagnose VSs and NF2. In the clinical model, the absence of an independent association of more recent year of diagnosis with a decrease in risk of mortality does not suggest that improvements in clinical care lack benefit. In all likelihood, insufficient time has elapsed for such benefits to be reflected in decreased risk of mortality. In addition, the benefits from incremental improvements in clinical care throughout the post-1970 era may be more subtle, with respect to risk of mortality, than those that occurred with the advent of computerized tomography scanning in the early 1970's.

Constitutional *NF2* mutations that are not found by SSCP could be mutations in the 3'or 5' UTRs, the promoter region, or untranscribed transcriptional control elements; intronic mutations that are not covered by SSCP primers; large deletions, insertions, or other rearrangements; or mutations in patients who are somatic mosaics. There is no evidence for locus heterogeneity in NF2.[37] In 60 United Kingdom NF2 families with two or more generations, all families have linkage to *NF2*, and *NF2* mutations have been identified in all but six families (D.G.R. Evans, unpublished data). Fifteen to twenty per cent of NF2 founders are thought to be somatic mosaics, and almost all have mild disease despite having constitutional nonsense or frameshift *NF2* mutations that typically cause severe disease in classical NF2.[19,20] We excluded known somatic mosaics from the analysis, and although some somatic mosaics probably were not detected, the resultant bias is likely to be minor. Of the 150 founders who underwent molecular screening, pathogenic *NF2* mutations were not identified in 65. Thirty-eight of these founders had an age at onset less than 20 years, or two or more meningiomas, or four or more spinal tumors. These patients are unlikely to be mosaic because they have severe disease.[19,20,38] The remaining 27 founders had mild disease. If 15-20% of them were mosaic, then we failed to identify only 4-5 mosaics in the screened group.

In summary, the strongest single predictor of the risk of mortality in NF2 is age at diagnosis, which is a useful index for patient counseling and clinical management. The presence of meningiomas is also useful in this regard. People with constitutional *NF2* missense mutations have lower risk of mortality than those with nonsense or frameshift mutations, but this was not as strong a predictor as the clinical covariates. Our finding that NF2 patients who are treated at specialty centers have a lower risk of mortality is consistent with studies of unilateral sporadic VS in which surgical outcomes were improved and operative complications were reduced in

proportion to increasing amount of surgical experience. We recommend that NF2 patients be referred to specialty centers for optimal care.

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Table 1. Characteristics of 350 NF2 patients in the United Kingdom registry

Characteristic[1]	Number	%
Vital status as of 1 August 2001		
Alive	276	78.9
Dead	74	21.1
Gender		
Female	179	51.1
Male	171	48.9
Inheritance		
Founder	209	59.7
Inherited case	141	40.3
Age at onset of symptoms (years)		
1-19	146	41.8
20-39	145	41.5
40-59	34	9.7
Asymptomatic at diagnosis	24	6.9
Age at diagnosis (years)		
1-19	111	31.7
20-39	175	50.0
40-59	59	16.9
≥ 60	5	1.4

Vestibular schwannomas		
None	23	6.6
Unilateral	34	9.8
Bilateral	289	83.6
Intracranial meningiomas		
Absent	190	54.8
Present	157	45.2
Type of constitutional *NF2* mutation		
Nonsense	37	13.7
Frameshift deletion	26	9.6
Frameshift insertion	10	3.7
Splice donor site	17	6.3
Splice acceptor site	33	12.2
Missense	22	8.1
Large deletion	46	17.0
In-frame deletion	1	0.0
Chromosomal rearrangement	2	0.1
Not identified	77	28.4
Type of treatment center		
Non-specialty	250	71.4
Specialty[2]	100	28.6

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[1]Four patients had unknown VS status, three had unknown meningioma status,

one had unknown age at onset of symptoms, and 77 had not been screened for mutations.

[2]Manchester Royal Infirmary, Addenbrooke's Hospital (Cambridge), and Royal London Hospital.

Table 2. Results of the clinical and molecular models for Cox regressions

Covariates and statistics	Clinical model Statistic	P	Molecular model Statistic	P
-2(log(likelihood))				
Total	248.3	< .001	128.7	< .001
Age at diagnosis	194.9		----	
Presence of meningiomas	19.6		----	
Type of treatment center	22.0		Need	
Calendar year of diagnosis	----		Need	
Missense mutations (compared to nonsense or frameshift mutations)	----		Need	
Schemper's v (95% CI)				
Per cent variance accounted for	54 (32 - 76)	< .001	Need	Need

The covariates in the clinical model were age at diagnosis, presence of meningiomas, and type of treatment center. The covariates in molecular model were type of constitutional *NF2* mutation, type of treatment center, and calendar year of diagnosis.

Figure legends

Median survival in univariate Kaplan-Meier survival curves (Wilcoxon [Gehan] statistic)

1. Age at diagnosis: < 20 years, 31 years; ≥ 20 years, 65 years ($P < .001$)

2. Intracranial meningiomas: present, 55 years; absent, 69+ years ($P = .008$)

3. Type of constitutional *NF2* mutation: nonsense or frameshift, 46 years; missense, 66+ years ($P = .006$)

4. Type of treatment center: non-specialty, 51 years; specialty, 66+ years ($P = .001$)

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Evaluation of clinical diagnostic criteria for neurofibromatosis 2

Michael E. Baser,[1] J. M. Friedman,[2] Andrew J. Wallace,[3]

Richard T. Ramsden,[4] Harry Joe,[5] D. Gareth R. Evans[3]

[1]Los Angeles, U.S.A., [2]Department of Medical Genetics and [5]Department of Biostatistics, University of British Columbia, Vancouver, Canada, [3]Department of Medical Genetics, St. Mary's Hospital, Manchester, U.K., [4]Department of Otolaryngology, Manchester Royal Infirmary, Manchester, U.K.

Address correspondence to:

Michael E. Baser, Ph.D.

2257 Fox Hills Dr., Los Angeles, CA 90064

e-mail baser@earthlink.net, tel 310-203-0351, fax 310-472-3614

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Article abstract- ***Objective***: To empirically evaluate the four existing sets of clinical diagnostic criteria for neurofibromatosis 2 (NF2). ***Background***: Each set of criteria was developed by a group of expert clinicians, but sensitivity has never been formally assessed. The four sets of criteria differ for people without bilateral vestibular schwannomas, which are pathognomonic for NF2. ***Methods***: The study was based on 163 of 403 people in the United Kingdom NF2 registry (41%) who presented without bilateral vestibular schwannomas. We applied the sets of criteria to each person at initial assessment and the most recent clinical evaluation (mean $\pm$ SE length of follow-up, 13 $\pm$ 1 years). ***Results***: In people with "definite NF2" and a negative family history of NF2, the 1987 U.S. National Institutes of Health (NIH) and 1991 NIH criteria each identify 78% of people at the most recent clinical evaluation, but 0% at initial assessment. The National Neurofibromatosis Foundation (NNFF) criteria and the Manchester criteria identify higher proportions at both time points (NNFF criteria, 91% and 10%; Manchester criteria, 93% and 14%), but the proportions at initial assessment are still low. ***Conclusions***: None of the existing sets of criteria is adequate at initial assessment for diagnosing people who present without bilateral vestibular schwannomas as having NF2, particularly people with a negative family history of NF2. We recommend that a single, revised set of diagnostic criteria be devised to replace all of the existing sets of criteria.

Introduction

Neurofibromatosis 2 (NF2) is an autosomal dominant disease, caused by inactivating mutations or loss of both alleles of the *NF2* tumor suppressor gene,[1,2] with an incidence of 1 in 33-40,000 live births.[3] Clinical features of NF2 typically include nervous system tumors (vestibular schwannomas, intracranial meningiomas, spinal tumors, and peripheral nervous system tumors), ocular abnormalities, and skin lesions.[4-6] In 1987, a U. S. National Institutes of Health (NIH) Consensus Conference established clinical diagnostic guidelines to differentiate NF2 from NF1.[7] Although the two diseases were then known to be genetically distinct,[8] their frequent confusion in the medical literature and the need to avoid misdiagnosis in linkage studies prompted the development of these criteria.

As the full clinical spectrum of NF2 became better defined by molecular analysis and neuroimaging, the 1987 NIH criteria proved to be too restrictive for use in routine diagnosis. Revisions to the criteria were recommended by a second NIH Consensus Conference in 1991,[9] the Manchester group in 1992,[4] and a group organized by the National Neurofibromatosis Foundation (NNFF) in 1997.[10] Each set of criteria was developed by a group of expert clinicians, but the sensitivity, specificity, and clinical utility of these diagnostic guidelines have never been formally assessed.

All four sets of criteria diagnose NF2 in people with bilateral vestibular schwannomas, and in people with a first-degree relative with NF2 and either a unilateral vestibular schwannoma at less than 30 years of age or at least two other characteristic disease features of NF2 (meningioma, non-vestibular schwannoma, glioma, presenile cataract). The diagnostic systems differ in other respects (Table 1). Half of all people with NF2 have a family history of the disease.[4]

The purpose of this study was to examine the diagnostic efficiency of the four sets of criteria in people who do not have bilateral vestibular schwannomas, but who do have other signs of NF2. We found that none of the existing sets of criteria is satisfactory for diagnosing such people at initial assessment. Overall and at the time of the most recent clinical evaluation, the Manchester criteria are the most sensitive.

Methods

The study population was selected from the United Kingdom NF2 registry. NF2 patients are ascertained by contacting neurosurgeons, otolaryngologists, neurologists, pediatricians, dermatologists, and geneticists throughout the United Kingdom. Patients are also identified through the Regional Cancer Registry in the North West Region. As of 1 April 2002, the registry had clinical and molecular information on 427 people with proven or suspected NF2, from 282 families. We excluded asymptomatic at-risk members of NF2 families who were diagnosed through genetic screening and did not have clinical information (N = 13), and 11 other people with insufficient clinical information for this study. Of the remaining 403 people, 240 (59%) had bilateral vestibular schwannomas at initial assessment.

This study was based on the 163 people (41%) who did not have bilateral vestibular schwannomas at initial assessment (108 new mutations and 55 inherited cases). Of these 163 people, 64 had left and right vestibular schwannomas diagnosed at the same time, but previously presented with other NF2-related abnormalities (meningioma, non-vestibular schwannoma, glioma, neurofibroma, presenile cataract); 42 had only a unilateral vestibular schwannoma during follow-up; 40 presented with a unilateral vestibular schwannoma but developed a contralateral vestibular schwannoma during follow-up; and 17 did not have any vestibular

schwannomas during follow-up. Of the 104 people who had bilateral vestibular schwannomas by the end of follow-up, left and right vestibular schwannomas were diagnosed at the same time in 64 people, but there was a delay of 1-5 years in 22 people, 6-10 years in 12 people, 11-15 years in four people, and more than 15 years in two people.

The NNFF criteria have separate categories for confirmed or definite NF2, and presumptive or probable NF2 (Table 1). For the purposes of this study, these two categories were considered to be equivalent. In new mutations from the United Kingdom NF2 registry who were screened for constitutional *NF2* mutations, mutations were found in 57% of 21 people who met the NNFF criteria for presumptive or probable NF2, a fraction similar to the 55% of 168 people with bilateral vestibular schwannomas.

At the end of follow-up, 59 people had a first-degree relative with NF2. Seventeen of these people did not have an affected first-degree relative at initial assessment. The affected first-degree relative was a parent in 53 patients and an offspring in six patients. In only one instance was the parent initially assessed after the offspring was diagnosed with NF2. An additional six people died before their first-degree relative was diagnosed with NF2. For the purposes of this study, these people were classified as not having an affected relative during follow-up, but this information was considered in determining inheritance.

The 163 people who did not have bilateral vestibular schwannomas at initial assessment were divided into two groups. People with "definite NF2" were those who met the specific criteria, by the end of follow-up, that all four sets of clinical diagnostic criteria have in common. Using each set of criteria, a person is diagnosed with NF2 if they have bilateral vestibular schwannomas, or a first-degree relative with NF2 and either a unilateral vestibular schwannoma at less than 30 years of age or two other characteristic NF2 disease feature types (meningioma,

non-vestibular schwannoma, glioma, presenile cataract). Identified constitutional *NF2* mutations were also used to define "definite NF2". One hundred forty-one people were classified as having "definite NF2": 104 had bilateral vestibular schwannomas by the end of follow-up, 98 had identified constitutional *NF2* mutations, eight had a first-degree relative with NF2 and a unilateral vestibular schwannoma at less than 30 years of age, and six had an affected first-degree relative, no vestibular schwannomas, but at least two other characteristic types of NF2 disease features. People with "possible NF2" (N = 22) had some characteristic features of NF2, but did not meet the criteria for "definite NF2". The length of follow-up was not significantly different between people with "definite NF2" and those with "possible NF2" (mean $\pm$ SE, 13 $\pm$ 1 years and 11 $\pm$ 2 years).

The four sets of diagnostic criteria were applied to these two groups to determine the proportion of people who met each set of criteria. We evaluated the diagnostic criteria at two time points: initial assessment and most recent clinical evaluation. The registry has the age at diagnosis of each tumor type and the age at which first-degree relatives were diagnosed, and for the analysis based on initial assessment we considered only those abnormalities and affected relatives that existed at that time point.

Using Kaplan-Meier analysis, we determined the time course, from initial assessment to the most recent clinical evaluation, of the increasing fraction of people who would be diagnosed with NF2 using the different sets of criteria as a result of new disease features developing or a first-degree relative being newly diagnosed. Only those manifestations and affected first-degree relatives that were present in each year of follow-up were considered. Because the Kaplan-Meier curves for the different sets of diagnostic criteria are based on the same people, we used

the jackknife method,[11] with family as the unit, to compute pointwise standard errors for differences in proportions of pairwise Kaplan-Meier curves.

Results

Table 2 presents the proportion of the 163 people who presented without bilateral vestibular schwannomas at initial assessment that were identified by each set of diagnostic criteria. At initial assessment, the 1987 NIH criteria and the 1991 NIH criteria each identified 0% of people with "definite NF2" and a negative family history of NF2, while the NNFF criteria and the Manchester criteria identified greater but still low proportions (10% and 14%). The 1991 NIH criteria identified 100% of people with "definite NF2" and a positive family history of NF2, while the other sets of criteria identified 45-69%. The 1987 NIH criteria and the 1991 NIH criteria did not identify any people with "possible NF2" as having NF2, while the NNFF criteria and the Manchester criteria identified greater but still low proportions (23% and 32%).

As expected, greater proportions of people were diagnosed with NF2 by each set of criteria at the most recent clinical evaluation than at initial assessment (i.e., after an average of 13 years from initial assessment). At the most recent clinical evaluation, the 1987 NIH criteria and the 1991 NIH criteria each identified 78% of people with "definite NF2" and a negative family history of NF2. The NNFF and Manchester criteria identified NF2 in higher proportions of these people (91% and 93%). All four sets of criteria identified high proportions of people with "definite NF2" and a positive family history of NF2 (96-100%). The Manchester criteria identified NF2 in 100% of people with "possible NF2", but the other sets of criteria identified NF2 in a much lower fraction (0-46%).

Figure 1 illustrates the proportion of the 121 people in this study with a negative family history of NF2 at initial assessment that met each of the four sets of diagnostic criteria with increasing length of time from initial assessment, using Kaplan-Meier analysis. At one year from initial assessment, the proportion of people that met the diagnostic criteria for NF2 was 27% for the Manchester criteria, 23% for the NNFF criteria, 5% for the 1991 NIH criteria, and 4% for the 1987 NIH criteria. At five years from initial assessment, these proportions were 49% for the Manchester criteria, 44% for the NNFF criteria, 27% for the 1991 NIH criteria, and 24% for the 1987 NIH criteria.

Table 3 presents the differences in proportions of people identified as having NF2 in comparisons between the different sets of diagnostic criteria (pointwise comparisons, corresponding to jump points in the Kaplan-Meier curves). For the Manchester criteria and the 1991 NIH criteria, the difference in proportions was significantly greater than zero from initial assessment to 25 years after initial assessment. For the NNFF criteria and the 1991 NIH criteria, the difference in proportions was significantly greater than zero from initial assessment to 15 years after initial assessment. For the Manchester criteria and the NNFF criteria, the difference in proportions was significantly greater than zero at initial assessment and from five to 20 years after initial assessment.

There were eight known somatic mosaics (determined through molecular analysis) among the 98 new mutations who were screened for constitutional *NF2* mutations. At initial assessment, only the Manchester criteria identified any of the mosaics (one) as having NF2. At the most recent clinical evaluation, the 1987 NIH criteria and 1991 NIH criteria each identified four mosaics as having NF2, the NNFF criteria identified five mosaics, and the Manchester criteria identified all eight mosaics. The median age at onset of symptoms in the eight mosaics

was 28 years (range, 23-46 years) and the median age at diagnosis was 43 years (range, 27-62 years). At initial assessment, four of the mosaics had unilateral vestibular schwannomas, two had cutaneous schwannomas, one had spinal tumors, and one had cataracts. At the most recent clinical evaluation, four of the mosaics had unilateral vestibular schwannomas, four had bilateral vestibular schwannomas, four had spinal tumors, two had cutaneous schwannomas, two had intracranial meningiomas, two had cataracts, and one had a non-VIII nerve cranial nerve tumor.

Discussion

Bilateral vestibular schwannomas are pathognomic for NF2, but 163 of the 403 people with clinical information in the United Kingdom NF2 registry (41%) did not have bilateral vestibular schwannomas at initial assessment. The four sets of clinical diagnostic criteria identify different proportions of these 163 people as having NF2, in both "definite NF2" and "possible NF2" patients. People with a negative family history of NF2 at initial assessment present the greatest diagnostic difficulties. Using Kaplan-Meier analysis, the Manchester criteria and the NNFF criteria each identify significantly higher proportions of these people than the 1991 NIH criteria, but the Manchester criteria usually identify a significantly higher proportion than the NNFF criteria.

The 1991 NIH criteria identify the highest proportion of people with "definite NF2" and a positive family history of NF2 because this set of criteria requires only one instead of two characteristic disease features to establish the diagnosis in people with a family history of the disease. However, the 1987 NIH criteria and the 1991 NIH criteria each require a positive family history to diagnose NF2 in people who do not have bilateral vestibular schwannomas. As a result, the NIH criteria identify the lowest proportion of people with "definite NF2" and a

negative family history of NF2, and few people with "possible NF2". The three people with "possible NF2" who were identified at the most recent clinical evaluation using the 1991 NIH criteria each had a family member who had been diagnosed with NF2 after initial assessment, no vestibular schwannomas or a vestibular schwannoma at 30 years of age or older, but one other characteristic disease feature of NF2.

Unlike the NIH criteria, a diagnosis of NF2 can be made using the Manchester criteria and the NNFF criteria in people who do not bilateral vestibular schwannomas or a family history of NF2, but who do have other characteristic disease features. The Manchester criteria and the NNFF criteria have similar wording, but the Manchester criteria identify a higher proportion of people than the NNFF criteria because the Manchester criteria are based on the number of disease features of any type (i.e., the number of individual tumors), while the NNFF criteria and the NIH criteria are based on the number of different feature types. For example, in a person with two intracranial meningiomas, the Manchester criteria count the two meningiomas as two disease features, while the NNFF criteria and the NIH criteria count the two meningiomas as a single disease feature because they are the same histologic type of tumor.

The Manchester criteria also differ from the NNFF criteria because the Manchester criteria do not require an age at diagnosis of unilateral vestibular schwannoma of less than 30 years. This age requirement decreases sensitivity and is not needed to increase specificity because people with unilateral vestibular schwannoma who do not have a family history of NF2 or any other characteristic disease features of NF2 have a very low probability of developing NF2. The probability decreases from 1% in people who are diagnosed with a unilateral vestibular schwannoma at ages 10-19 years, to 0.45% in those aged 20-29 years, to 0.15% in those aged 30-39 years.[12] Figure 2 presents data from the United Kingdom showing that 54% of

NF2 new mutations are diagnosed with their first vestibular schwannoma at less than 30 years of age compared to only 5% of people with unilateral vestibular schwannomas, but conversely, 46% of NF2 new mutations are diagnosed with their first vestibular schwannoma at 30 years of age and older. Screening for constitutional *NF2* mutations in the small fraction of people with unilateral vestibular schwannoma who present at less than 30 years of age can identify people with NF2.[12-14]

In the present study, the NNFF age requirement reduces sensitivity. Due solely to the age requirement, 14 people who were diagnosed with unilateral vestibular schwannomas at 30 years of age or older are not identified by the NNFF criteria, but are identified by the Manchester criteria. There are four people with "definite NF2", including three somatic mosaics, and 10 people with "possible NF2". In most NF2 somatic mosaics, disease manifestations are milder and vestibular schwannomas are of later onset than in people with classical NF2.[15-17] The odds of somatic mosaicism in NF2 increase 11-fold per decade increase in age at diagnosis of NF2, and are 7-fold greater in NF2 patients with no vestibular schwannomas or a unilateral vestibular schwannoma compared to those with bilateral vestibular schwannomas.[17]

A "gold standard" does not exist for distinguishing normal individuals from those who actually have NF2 in every case. The present study focuses on sensitivity, but there is considerable evidence that specificity is high (about 99%), and probably similar for each set of diagnostic criteria.[12-14] As noted above, people with unilateral vestibular schwannoma who do not have a family history of NF2 or characteristic disease features of NF2 have a very low probability of developing NF2, and this probability decreases with increasing age at diagnosis of vestibular schwannoma.[13,14] Sporadic unilateral vestibular schwannoma can occur in two first-degree relatives by chance, giving the appearance of heritable vestibular schwannomas.

Sporadic unilateral vestibular schwannoma has a lifetime prevalence of about 1 in 1,000, and assuming that people have an average of five first-degree relatives, the lifetime probability of sporadic unilateral vestibular schwannoma occurring by chance in two first-degree relatives is 0.5%.[12]

Two people in this study are of particular interest because of their late age at first vestibular schwannoma. They were diagnosed with their first vestibular schwannoma at ages 60 and 72, and each developed a contralateral vestibular schwannoma two years later. Neither had a family history of NF2 nor any other clinical feature of the disease. One person had molecular testing and was found to be mosaic for a constitutional frameshift mutation. The other person also might be a somatic mosaic, or a rare person with two sporadic unilateral vestibular schwannomas who does not have NF2 (this is most likely in older people, in whom the incidence of unilateral sporadic vestibular schwannoma is highest[18]). A third possibility is that the person might have non-mosaic NF2 with unusually mild expression, perhaps as a result of a "weak" mutant allele. The two adult children of the patient do not have clinical features of NF2; in such instances, family molecular genetic studies may be useful.

A more sensitive set of diagnostic criteria for NF2 can be developed by adding mononeuropathy as a clinical diagnostic criterion and incorporating the results of genetic testing.[19] In the United Kingdom NF2 registry, mononeuropathy (foot drop, wrist drop, or facial palsy) occurs in 31% of people with an age at onset of symptoms of less than 15 years, but in only 3% of those with an age at onset of 15 years or older. NF2-associated mononeuropathy has been reported in previous studies,[20-24] as has a more generalized peripheral neuropathy.[4,5,25] The differential diagnosis should exclude polio.[4] In general, the presenting symptom in young people with NF2 is less likely to be vestibular than in adult NF2 patients.[22,26,27]

NF2 is 99% penetrant by age 60,[28] and genetic testing to identify pathogenic constitutional *NF2* mutations or *NF2* mutation carriers can increase sensitivity with minimal decrease in specificity.[19] Negative results from single-strand conformational polymorphism (SSCP) analysis on blood samples, a commonly-used method,[29] cannot rule out NF2 because such testing would not identify mutations in the 3 or 5 untranslated regions, the promoter region, untranscribed transcriptional control elements, or introns that are not covered by conventional SSCP primers; large deletions, insertions, or other rearrangements; or mutations in patients who are somatic mosaics if the mutant line is not sufficiently abundant in the tissue tested.[30] Other epigenetic events (i.e., methylation) also could result in loss of *NF2* expression.[31] Segregation analysis using tightly-linked genetic markers can identify *NF2* mutation carriers in at-risk members of NF2 families. Segregation analysis is more rapid and less expensive than mutation analysis, and in conjunction with clinical information, often can discriminate between *NF2* mutation carriers and non-carriers with a high degree of certainty.[32,33]

We recommend that a single, revised set of diagnostic criteria be devised to replace all of the existing diagnostic systems for NF2. In future work, we will empirically validate our suggestions for revised criteria.

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Table 1. Clinical diagnostic criteria for NF2

1987 NIH criteria

A. Bilateral vestibular schwannomas

B. 1st degree family relative with NF2 *and* unilateral vestibular schwannoma *or* any two of: meningioma, schwannoma, glioma, neurofibroma, juvenile posterior subcapsular lenticular opacity

1991 NIH criteria

A. Bilateral vestibular schwannomas

B. 1st degree family relative with NF2 *and* unilateral vestibular schwannoma *or* any one of: meningioma, schwannoma, glioma, neurofibroma, juvenile posterior subcapsular lens opacity

Manchester criteria

A. Bilateral vestibular schwannomas

B. 1st degree family relative with NF2 *and* unilateral vestibular schwannoma *or* any two of: meningioma, schwannoma, glioma, neurofibroma, posterior subcapsular lenticular opacities

C. Unilateral vestibular schwannoma *and* any two of: meningioma, schwannoma, glioma, neurofibroma, posterior subcapsular lenticular opacities

D. Multiple meningiomas (two or more) *and* unilateral vestibular schwannoma *or* any two of: schwannoma, glioma, neurofibroma, cataract

(Note: In the Manchester criteria, "any two of" refers to two individual tumors or cataract, while in the other sets of criteria, it refers to two tumor types or cataract)

NNFF criteria

1. Confirmed or definite NF2

A. Bilateral vestibular schwannomas

B. 1st degree family relative with NF2 *and* unilateral vestibular schwannoma at less than 30 years of age *or* any two of: meningioma, schwannoma, glioma, juvenile lens opacity (posterior subcapsular cataract or cortical cataract)

2. Presumptive or probable NF2

A. Unilateral vestibular schwannoma at less than 30 years of age *and* at least one of: meningioma, schwannoma, glioma, juvenile lens opacity (posterior subcapsular cataract or cortical cataract)

B. Multiple meningiomas (two or more) *and* unilateral vestibular schwannoma at less than 30 years of age *or* at least one of: schwannoma, glioma, juvenile lens opacity (posterior subcapsular cataract or cortical cataract)

(Note: For the purposes of this study, the NNFF criteria for confirmed or definite NF2 and for presumptive or probable criteria were considered to be equivalent)

Table 2. Four sets of NF2 diagnostic criteria applied to 163 people in the United Kingdom NF2 registry who presented without bilateral vestibular schwannomas, by time of evaluation, disease group, and family history

Diagnostic criteria[1]	Number (%) of patients identified by time of evaluation, disease group,[2] and family history[3]					
	Initial assessment			Most recent clinical evaluation[4]		
	"Definite NF2"		"Possible NF2"	"Definite NF2"		"Possible NF2"
	Family history			Family history		
	Negative (N = 99)	Positive (N = 42)	(N = 22)	Negative (N = 85)	Positive (N = 56)	(N = 22)
1987 NIH	0 (0)	19 (45)	0 (0)	66 (78)	55 (98)	0 (0)
1991 NIH	0 (0)	42 (100)	0 (0)	66 (78)	56 (100)	3 (14)
NNFF	10 (10)	17 (40)	5 (23)	77 (91)	54 (96)	10 (46)
Manchester	14 (14)	29 (69)	7 (32)	79 (93)	55 (98)	22 (100)

[1]See Table 1 for definition of the different sets of diagnostic criteria.

[2]17 first-degree relatives of people with "definite NF2" were diagnosed after initial assessment (14 "definite NF2"patients and three "possible NF2" patients).

[3]"Definite NF2": people who had bilateral vestibular schwannomas by the end of follow-up, or who had a first-degree relative with NF2 and either a unilateral vestibular schwannoma at less than 30 years of age or at least two other characteristic disease feature types of NF2, or who had an identified constitutional *NF2* mutation. "Possible NF2": people who had some characteristic disease features of NF2, but who did not meet the criteria for "definite NF2".

[4]The mean $\pm$ SE period of follow-up was 13 $\pm$ 1 years.

Table 3. Pointwise comparisons of Kaplan-Meier curves in Figure 1, difference in proportions of people identified as having NF2 using different sets of diagnostic criteria, by time since initial assessment

Time since initial assessment (years)	Comparisons between sets of diagnostic criteria					
	Manchester v. 1991 NIH		NNFF v. 1991 NIH		Manchester v. NNFF	
	Difference in proportions	95% CI	Difference in proportions	95% CI	Difference in proportions	95% CI
0	0.174	0.107 - 0.241	0.124	0.065 - 0.183	0.050	0.005 - 0.095
1	0.215	0.141 - 0.289	0.174	0.101 - 0.247	0.040	0.000 - 0.089
2	0.230	0.154 - 0.306	0.190	0.114 - 0.266	0.039	0.000 - 0.086
3	0.236	0.160 - 0.312	0.197	0.121 - 0.273	0.039	0.000 - 0.086
4	0.240	0.158 - 0.322	0.193	0.113 - 0.273	0.046	0.000 - 0.093
5	0.233	0.139 - 0.307	0.170	0.088 - 0.252	0.053	0.010 - 0.096
6	0.234	0.148 - 0.320	0.175	0.093 - 0.257	0.060	0.015 - 0.105
7	0.228	0.144 - 0.312	0.170	0.088 - 0.252	0.058	0.015 - 0.101

8	0.191	0.113 - 0.269	0.126	0.048 - 0.204	0.065	0.020 - 0.110
9	0.196	0.125 - 0.270	0.133	0.059 - 0.207	0.063	0.020 - 0.106
10	0.195	0.119 - 0.271	0.118	0.045 - 0.191	0.078	0.031 - 0.125
11	0.221	0.141 - 0.301	0.148	0.070 - 0.226	0.074	0.029 - 0.119
12	0.199	0.123 - 0.275	0.124	0.050 - 0.198	0.075	0.030 - 0.120
13	0.177	0.103 - 0.251	0.116	0.043 - 0.189	0.061	0.014 - 0.118
14	0.158	0.087 - 0.229	0.100	0.031 - 0.169	0.059	0.012 - 0.116
15	0.151	0.080 - 0.222	0.085	0.020 - 0.150	0.066	0.023 - 0.107
16	0.140	0.064 - 0.216	0.069	0.000 - 0.107	0.071	0.020 - 0.122
17	0.144	0.066 - 0.222	0.077	0.001 - 0.153	0.067	0.016 - 0.118
18	0.135	0.064 - 0.206	0.073	0.004 - 0.142	0.061	0.012 - 0.110
19	0.123	0.056 - 0.192	0.069	0.000 - 0.138	0.054	0.070 - 0.101
20	0.118	0.049 - 0.187	0.067	0.000 - 0.136	0.050	0.003 - 0.097
21	0.111	0.042 - 0.180	0.067	0.000 - 0.136	0.044	0.000 - 0.089
22	0.095	0.030 - 0.160	0.051	0.000 - 0.116	0.044	0.000 - 0.089
23	0.062	0.009 - 0.115	0.018	0.000 - 0.073	0.044	0.000 - 0.089
24	0.058	0.003 - 0.113	0.034	0.000 - 0.097	0.024	0.000 - 0.061

25	0.060	0.009 - 0.111	0.049	0.000 - 0.104	0.011	0.000 - 0.042
30	0.052	0.000 - 0.148	0.017	0.000 - 0.117	0.035	0.000 - 0.074

CI = confidence interval. Differences in proportions and 95% CIs are constant in the interval between 25 and 30 years after initial assessment.

Figure legends

Figure 1. Proportion of 121 people with a negative family history of NF2 at initial assessment that meet the four sets of clinical diagnostic criteria with increasing length of time from initial assessment (Kaplan-Meier analysis). Solid line = Manchester criteria. Dashed line = NNFF criteria. Dashed-dotted line = 1991 NIH criteria. Dotted line = 1987 NIH criteria.

Figure 2. Distribution of age at diagnosis of 481 people with unilateral vestibular schwannoma in the St. Mary's Hospital geographic catchment area (Manchester, United Kingdom), and age at first vestibular schwannoma of 127 NF2 new mutations in the United Kingdom NF2 registry (time period for both groups, 1987-1997). People with known NF2 are excluded from the people with unilateral vestibular schwannoma, and known NF2 somatic mosaics (defined through molecular testing) are excluded from the NF2 new mutations. The median age at diagnosis for people with unilateral vestibular schwannoma was 53 years, and the median age at first vestibular schwannoma for NF2 new mutations was 29 years. Solid bars = first NF2 vestibular schwannoma. Cross-hatched bars = unilateral vestibular schwannoma.

ANALYSIS OF NEUROFIBROMATOSIS 1 (NF1) LESIONS BY BODY SEGMENT

Vairochana Palmer[1,2], Jacek Szudek[1,3], Harry Joe[4], Vincent M. Riccardi[5], and J.M. Friedman[1,*]

[1] *Department of Medical Genetics, The University of British Columbia, Vancouver, CANADA*

[2] *Department of Genetics, Stanford University, Stanford, California, USA*

[3] *Dalhousie Medical School, Halifax, Nova Scotia, CANADA*

[4] *Department of Statistics, The University of British Columbia, Vancouver, CANADA*

[5] *American Medical Consumers, La Crescenta, California, USA*

Running Title: Analysis of NF1 lesions by body segment

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* Correspondence to: J. M. Friedman, MD, PhD; Department of Medical Genetics; The University of British Columbia; 6174 University Boulevard; Vancouver, BC V6T 1Z3; CANADA

Telephone: (604) 822-2749

Fax: (604) 822-5348

E-mail: frid@interchange.ubc.ca

ABSTRACT

Café-au-lait spots and neurofibromas are defining features of neurofibromatosis 1 (NF1), but they vary greatly in number, size, and clinical importance from patient to patient. The cause of this variability is unknown. We tested the hypotheses that development of these lesions is influenced by local or familial factors.

The presence or absence of café-au-lait spots, cutaneous neurofibromas, and diffuse plexiform neurofibromas was recorded for each of ten divisions of the body surface in 547 NF1 patients, including 117 affected individuals in 52 families. We used stratified Mantel-Haenszel tests to look for local associations between the presence of diffuse plexiform neurofibromas, cutaneous neurofibromas, and café-au-lait spots in individual body segments of NF1 patients. We used a random effects model to obtain intrafamilial correlation coefficients for the age-adjusted number of body divisions affected with each of the three lesions.

No significant association was observed between the occurrence of cutaneous and diffuse plexiform neurofibromas, between café-au-lait spots and cutaneous neurofibromas, or between café-au-lait spots and plexiform neurofibromas in the same body segment. The correlation among relatives in the number of body segments affected with café-au-lait spots was 0.45 (95% confidence interval [CI] = 0.18, 0.71), with cutaneous neurofibromas, 0.37 (95% CI = 0.15, 0.55), and with plexiform neurofibromas, 0.35 (95% CI = 0.15, 0.57). We conclude that the development of café-au-lait spots, cutaneous neurofibromas, and plexiform neurofibromas are spatially independent in NF1 patients but that the development of all three lesions is influenced by familial factors.

Keywords: Neurofibromatosis 1, familial correlation, café-au-lait spots, neurofibromas

INTRODUCTION

Neurofibromatosis 1 (NF1) is an autosomal dominant condition characterized by extremely variable expressivity. Café-au-lait spots and neurofibromas are the defining features. Neurofibromas are complex benign tumors arising in the fascicles of peripheral nerves (Korf, 1999). Histologically, a local increase in endoneurial matrix of the fascicle is accompanied by a thickened perineurium, increased size and number of Schwann cells (Harkin and Reed, 1969; Woodruff, 1999), and increased numbers of mast cells and fibroblasts (Giorno et al., 1989). Cutaneous neurofibromas are confined to a single fascicle within a nerve, while diffuse plexiform neurofibromas involve multiple fascicles (Burger and Scheithauer, 1994).

Cutaneous neurofibromas begin to appear in mid-childhood and eventually develop in almost all NF1 patients (Friedman and Riccardi, 1999; DeBella et al., 2000). Cutaneous neurofibromas tend to increase in number and size with age. Some adults with NF1 have hundreds or thousands of these lesions; other NF1 patients develop only a few cutaneous neurofibromas throughout life.

Diffuse plexiform neurofibromas are almost always, if not always, congenital (Friedman and Riccardi, 1999). Many are apparent on surface examination, although they often extend into deeper tissues. Some diffuse plexiform neurofibromas involve only deeper tissues and are not apparent on physical examination. Plexiform neurofibromas tend to be larger than cutaneous neurofibromas, sometimes involving an entire limb or other part of the body. Plexiform neurofibromas may give rise to malignant peripheral nerve sheath tumours, but discrete cutaneous neurofibromas rarely, if ever, do.

Café-au-lait spots are pigmented macules. Histologically, they contain melanocytes with abnormally large pigment particles (Fitzpatrick, 1981). Café-au-spots may be present at birth,

and by one year of age almost all children with NF1 have 6 or more of these lesions (Friedman and Riccardi, 1999; DeBella et al., 2000).

The number and location of café-au-lait spots and neurofibromas are highly variable, even among NF1 patients of similar age. The cause of this variability is unknown. Here we test the hypotheses that the development of these lesions is influenced by local or familial factors.

SUBJECTS AND METHODS

Subjects. 547 NF1 patients, including 117 affected individuals in 52 families, who had information recorded on spatial distribution of skin lesions were available in the NF Institute Database (Riccardi 1992). All of these patients were evaluated by Dr. Vincent Riccardi, and all meet the NIH diagnostic criteria for NF1 (Gutmann et al. 1997; National Institutes of Health Consensus Development Conference 1988). For each patient, the presence of one or more café-au-lait spots, one or more cutaneous neurofibromas, and one or more diffuse plexiform neurofibromas was recorded for each of the ten divisions of the body surface shown in Figure 1.

Analysis of local effect. We used two-layered Mantel-Haenszel tests (SPSS 1998) to look for local associations between the presence of diffuse plexiform neurofibromas and cutaneous neurofibromas in individual body segments of each NF1 patient. We stratified simultaneously by the body segment being considered and by the number of other body segments with one or more cutaneous neurofibromas (a categorical variable with range 0 to 9). This stratification was used to adjust for the fact that an NF1 patient who has a larger total number of body segments

with one or more neurofibromas is more likely to have at least one neurofibroma in any particular segment than an NF1 patient who has fewer total body segments affected. Confidence intervals for the summary odds ratio were obtained using a jackknife based on 20 different subgroups – a number that is sufficiently large to produce a stable estimate (Miller 1974). Homogeneity was assessed using the Breslow-Day test (SPSS 1998). Local associations between café-au-lait spots and cutaneous neurofibromas and between café-au-lait spots and plexiform neurofibromas were analyzed in the same manner.

Skin surface area. The body divisions used in this study cover varying amounts of skin surface area, so we checked for an association between the surface area and the presence of one or more cutaneous neurofibromas in a segment. Using logistic regression, we set the segment area as the independent variable and the presence or absence of cutaneous neurofibromas as the dependent variable. We tested in a similar manner for associations between surface area and the presence of diffuse plexiform neurofibromas and café-au-lait spots in a segment. Since the median age of our patients was 13 years, we approximated the surface area of the body segments by using standard percentages for 10-14 year-old individuals (McManus and Pruitt 1996). The proportions of total surface area assigned to each body segment were: head = 11%, neck = 2%, right upper torso = 12%, left upper torso = 12%, right lower torso = 4%, left lower torso = 4%, right arm = 9.5%, left arm = 9.5%, right leg = 18%, and left leg = 18%.

Total number of neurofibromas. In addition to data on whether each body segment was affected by one or more cutaneous neurofibromas, complete counts of cutaneous neurofibromas were available for 44 of the patients. The total number of neurofibromas in these patients ranged

from none to several hundred and appeared to increase logarithmically with the number of affected segments. We used linear regression (SPSS 1998) to test the relationship between log-transformed counts of the total number of cutaneous neurofibromas in an individual and the number of body segments that included one or more cutaneous neurofibromas. Counts of the total number of café-au-lait spots were not made, and few subjects had more than one plexiform neurofibroma, so these variables were not analyzed in this manner.

Familial analysis. For the familial analysis, we stratified subjects into 5-year age intervals, calculated the deciles for the total number of segments affected with cutaneous neurofibromas in each stratum, and ranked each subject by decile for the stratum in which he or she lay. We then used random effects models to obtain maximum likelihood estimates and confidence intervals for intrafamilial correlation coefficients for rank (Donner et al. 1989; Spjotvoll 1967). Café-au-lait spots and plexiform neurofibromas were analysed in the same manner.

RESULTS

We studied the distribution of café-au-lait spots, cutaneous neurofibromas, and diffuse plexiform neurofibromas in 10 segments of the body surface (Figure 1) in each of 547 patients with NF1. Two hundred eighty-one (51.4%) of the subjects were female, and 266 (48.6%) were male. Four hundred twenty-six (77.9%) were white, 67 (12.2%) were Hispanic, 44 (8.0%) were black and 10 (1.8%) were of other or mixed origin. Mean age was 17.5 years, and median age was 13 years.

Lesion frequency by body segment. Table 1 shows the frequency of these lesions in each of the 10 body segments. Two hundred ten patients had no cutaneous neurofibromas in any segment, and 337 patients had one or more cutaneous neurofibromas. Plexiform neurofibromas were noted in 216 patients. Cutaneous and plexiform neurofibromas occurred with similar frequencies in all ten body segments. Café-au-lait spots were observed in almost all patients and had similar frequencies in all segments except the head, where these lesions were less frequent.

No associations between lesion types within individual body segments. Table 2 shows the ten body segments examined and the odds ratios for associations of each pair of lesions for each segment. No association was observed between the occurrence of cutaneous and diffuse plexiform neurofibromas in the same body segment. The summary odds ratio was 1.20 (95% confidence interval [CI] = 0.81, 1.79). There was no evidence for heterogeneity across body segments (p=0.37).

Similarly, there was no association between the presence of café-au-lait spots and either cutaneous or diffuse plexiform neurofibromas within a single body segment. The summary odds ratios were 1.26 (95% CI = 0.82, 1.93) for café-au-lait spots and cutaneous neurofibromas and 1.25 (95% CI = 0.74, 2.12) for café-au-lait spots and plexiform neurofibromas. There was significant (p=0.03) heterogeneity in the occurrence of cutaneous neurofibromas and café-au-lait spots, with a positive association seen in the neck (odds ratio=2.94; 95% CI = 1.20, 7.20). No evidence of heterogeneity across body segments was found for the occurrence of plexiform neurofibromas and café-au-lait spots (p=0.52).

Log-linear relationship between segment size and number of cutaneous neurofibromas. The number of body segments affected with one or more cutaneous neurofibromas was strongly correlated with the total number of cutaneous neurofibromas in 44 NF1 patients in whom both total counts and data on the number of affected body segments were available ($r=0.95$, $p<0.001$). The relationship is log linear; the regression equation is

$$Log(\text{total number of neurofibromas} + 1) = 0.23 * (\text{number of segments affected}) + 0.014.$$

We observed no significant association between the relative size of the body surface area in a segment and the presence of one or more cutaneous neurofibromas ($p=0.18$) or of a diffuse plexiform neurofibroma ($p=0.23$). In contrast, an association was found between the presence of one or more café-au-lait spot in a body segment and its surface area expressed as a percentage of the body's total ($p<0.001$, odds ratio = 1.030, 95% CI = 1.015, 1.046).

All three lesions are correlated among relatives with NF1. We estimated intrafamilial correlations in the age-adjusted number of body segments that included one or more café-au-lait spots, one or more cutaneous neurofibromas, or one or more plexiform neurofibromas in 117 affected members of 52 families. We found significant intrafamilial correlations for the number of body segments affected by each of these clinical features. The intrafamilial correlation coefficient for the number of body segments affected with café-au-lait spots was 0.45 (95% CI = 0.18, 0.71). The correlation among relatives with NF1 for the number of body segments affected with cutaneous neurofibromas was 0.37 (95% CI = 0.15, 0.55). The correlation coefficient

among relatives for the number of body segments affected with plexiform neurofibromas was 0.35 (95% CI = 0.15, 0.57).

DISCUSSION

Lesions in body segments of individual patients. The number of body segments affected by one or more cutaneous neurofibromas appears to provide a good measure of how severely each of these NF1 patients is affected by this disease feature. We found a very high correlation (r = 0.95) between the number of body segments in which one or more cutaneous neurofibromas was present and the total number of cutaneous neurofibromas in 44 patients in whom counts were available. It seems likely that a similar relationship exists between the number of body segments affected with café-au-lait spots or plexiform neurofibromas and the severity of each of these disease features, but we did not have information on total counts of these lesions available to demonstrate this.

We have shown previously that individuals with diffuse plexiform neurofibromas are more likely also to have dermal neurofibromas (Szudek et al. 2000a; Szudek et al. Submitted for publication-a), but this association did not take into account the location or number of these lesions. The current study is the first to examine this association within body divisions. Since almost all, if not all, diffuse plexiform neurofibromas are of congenital origin (Friedman and Riccardi 1999), we wanted to find out if they influence the subsequent development of cutaneous neurofibromas. Our findings indicate that the occurrence of cutaneous neurofibromas in NF1 patients is not strongly influenced by the local presence of a diffuse plexiform neurofibroma. In

fact, we found that all three of the lesions studied (café-au-lait spots, cutaneous neurofibromas, and plexiform neurofibromas) occurred independently of each another in almost all of the body segments analyzed (Table 2).

We found a significant association between café-au-lait spots and cutaneous neurofibromas only in the neck. One possible reason the neck might be affected by both lesions is recurrent minor trauma to the skin associated with flexion, extension, and rotation of the head (Riccardi 1990). Clearly, however, other factors are also involved in the pathogenesis of café-au-lait spots and neurofibromas, as indicated by the familial correlations we observed for the age-adjusted number of body segments affected by each of the three lesions studied.

Familial correlations. The intrafamilial correlations we observed for cutaneous neurofibromas and café-au-lait spots in NF1 patients are consistent with the findings of a previous study (Easton et al. 1993). The number of familial patients and the prevalences of all three lesions were similar in these two studies. Our study found a similar correlation for café-au-lait spots but higher correlation coefficients for cutaneous neurofibromas than Easton and his associates did. We also found a significant familial correlation for plexiform neurofibromas. Easton et al. only analyzed this feature as a discrete (present/absent) trait and found no familial association.

We have also studied the familiality of café-au-lait spots, cutaneous neurofibromas, and plexiform neurofibromas as discrete traits in an independent series of NF1 patients using multivariate probit regression analysis with adjustment for age and the presence of associated clinical features (Szudek et al. 2000b; Szudek et al. Submitted for publication-b). The results of that study are consistent with the current one and with the study of Easton and associates (1993) despite the differences in design and methodology: We again found strong intrafamilial

correlations for café-au-lait spots (r = 0.43, 95% CI 0.29-0.57) and cutaneous neurofibromas (r = 0.49, 95% CI 0.33-0.65). Like Easton et al., we did not find a correlation for the occurrence of plexiform neurofibromas considered as a discrete trait when all relatives were considered, but we did find a significant sib-sib correlation for the occurrence of this clinical feature (r = 0.18, 95% CI 0.04-0.32). These observations provide further evidence for the importance of familial factors in the development of café-au-lait spots and neurofibromas in people with NF1.

The genetic basis for these familial associations has not been determined, but contributing factors may include effects of the mutant *NF1* allele itself, effects of the normal *NF1* allele, or modifying effects of other loci. The moderate magnitudes of the intrafamilial correlation coefficients show that familial factors alone are insufficient to predict the degree to which a patient will be affected by these lesions.

Our results are consistent with the possibility that different pathogenic mechanisms are involved in development of the three lesions studied. Chimeric mice composed in part of $Nf1^{-/-}$ cells develop plexiform neurofibromas but not cutaneous neurofibromas (Cichowski et al. 1999; Vogel et al. 1999). On the other hand, insertion of *tax* into the germline of mice leads to the development of multiple cutaneous neurofibromas but not plexiform neurofibromas (Feigenbaum et al. 1996). It is, therefore, clear that these two types of neurofibromas can develop by independent pathways, at least in mice. Some families with *NF1* mutations develop café-au-lait spots but no tumours (Abeliovich et al. 1995), consistent with different pathogenic factors being involved in the development of café-au-lait spots and neurofibromas.

In summary, multiple factors appear to be involved in the pathogenesis of café-au-lait spots as well as of both plexiform and cutaneous neurofibromas in patients with NF1. Some of

these factors are familial, but others are not. Some pathogenic factors may be shared among these three lesions, but other pathogenic mechanisms appear to differ.

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Table 1: Number and percentage of 547 NF1 patients who have one or more cutaneous neurofibromas, diffuse plexiform neurofibromas or café-au-lait spots in each of 10 body segments.

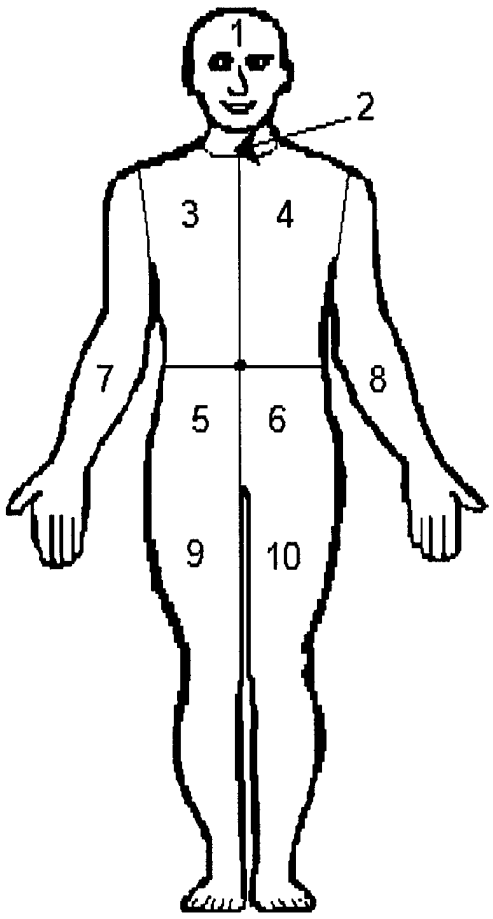
Segment	Cutaneous Neurofibromas		Plexiform Neurofibromas		Café-au-lait Spots	
	Total	(%)	Total	(%)	Total	(%)
1 Head	179	(33%)	47	(8%)	101	(18%)
2 Neck	168	(31%)	29	(5%)	397	(73%)
3 Right Upper Torso	259	(47%)	32	(6%)	532	(97%)
4 Left Upper Torso	258	(47%)	21	(4%)	531	(97%)
5 Right Lower Torso	285	(52%)	55	(10%)	537	(98%)
6 Left Lower Torso	287	(52%)	41	(7%)	533	(97%)
7 Right Arm	206	(38%)	21	(4%)	514	(94%)
8 Left Arm	208	(38%)	19	(3%)	511	(93%)
9 Right Leg	219	(40%)	54	(10%)	527	(96%)
10 Left Leg	220	(40%)	45	(8%)	525	(96%)
Total	***337***	***(62%)***	***216***	***(39%)***	***543***	***(99%)***

Table 2: Associations between cutaneous neurofibromas, diffuse plexiform neurofibromas and café-au-lait spots by body segment in 547 NF1 patients. Odds ratios could not be calculated for the association of café-au-lait spots and plexiform neurofibibromas in the right upper torso, right lower torso, or right arm because there were no patients who had plexiform neurofibromas but did not have café-au-lait spots in these segments.

	Cutaneous and Plexiform Neurofibromas		Cutaneous Neurofibromas and Café-au-lait Spots		Café-au-lait spots and Plexiform Neurofibromas	
Segment	**Odds Ratio**	**(95% C.I.)**	**Odds Ratio**	**(95% C.I.)**	**Odds Ratio**	**(95% C.I.)**
1 Head	0.95	(0.34-2.68)	1.34	(0.67-2.67)	1.26	(0.60-2.65)
2 Neck	2.39	(0.51-11.20)	2.59	(1.23-5.47)	2.42	(0.71-8.24)
3 Right Upper Torso	0.83	(0.23-3.02)	0.26	(0.01-10.34)	-	-
4 Left Upper Torso	0.39	(0.06-2.49)	0.12	(0.01-7.07)	1.29	(0.02-83.37)
5 Right Lower Torso	0.85	(0.32-2.24)	0.98	(0.01-84.41)	-	-
6 Left Lower Torso	0.91	(0.35-2.36)	1.13	(0.19-6.94)	0.06	(0.01-0.99)
7 Right Arm	1.17	(0.09-14.43)	1.91	(0.29-12.67)	-	-
8 Left Arm	1.01	(0.18-5.60)	0.91	(0.18-4.65)	0.22	(0.02-1.97)
9 Right Leg	3.91	(1.02-15.06)	0.2	(0.04-1.15)	0.35	(0.05-2.34)
10 Left Leg	3.6	(0.99-13.08)	1.1	(0.24-5.00)	2.7	(0.17-44.12)
Summary	***1.2***	***(0.81-1.79)***	***1.36***	***(0.91-2.03)***	***1.25***	***(0.74-2.12)***

FIGURE LEGEND

Figure 1: Body segment scheme used by Neurofibromatosis Institute Database.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Exploring the '2-Hit Hypothesis' In NF2: Tests of 2-hit and 3-hit Models of Vestibular Schwannoma Development

Ryan Woods[1], J. M. Friedman[2], D.Gareth R Evans[3], Michael E. Baser[4], and Harry Joe[1]

[1]Departments of Statistics and [2]Medical Genetics, University of British Columbia, Vancouver, Canada; [3]St. Mary's Hospital, Manchester, U.K; [4]Los Angeles, U.S.A.

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Corresponding author:

J.M. Friedman, M.D., Ph.D.
Department of Medical Genetics
University of British Columbia
6174 University Blvd, Room 228
Vancouver, BC, Canada V6T 1Z3
Telephone: 604-822-0367
Fax: 604-822-5348
E-mail: frid@interchange.ubc.ca

ABSTRACT

Neurofibromatosis 2 (NF2) is a genetic disease that affects approximately 1 in 40,000 people. Almost all affected individuals develop bilateral tumors of Schwann cells that surround the vestibular nerves; these tumors are called as vestibular schwannomas (VS). Evidence from molecular genetic studies suggests that at least two mutations are involved in formation of VS in patients with NF2. Several authors have proposed probabilistic models for this process in other tumors and have shown that such models are consistent with incidence data.

We have evaluated two different probabilistic models for a "2-hit" hypothesis for VS development in NF2 patients and present results from fitting these models to incidence data. Molecular evidence does not exclude the possibility that additional hits are necessary for development of VS, and we also assessed a "3-hit" model for tumor formation. The "3-hit" model fits the data marginally better than one of the "2-hit" models and much better than the other "2-hit" model. Our findings suggest that more than two mutations may be necessary for VS development in NF2 patients.

INTRODUCTION

Probabilistic models for tumorigenesis have been used extensively in genetic epidemiology to generate and test hypotheses about the genetic mechanisms that are responsible for tumor development [Armitage and Doll, 1954; Hethcote and Knudson, 1978; Knudson, 1971; Moolgavkar and Knudson, 1981; Moolgavkar and Luebeck, 1992]. The common theme incorporated into most of these models is that a tumor cell is assumed to be the outcome of a sequence of irreversible events (mutations). These events progressively transform normal tissue cells into tumor cells. Chu [1987] provides a clear, non-mathematical introduction to these models.

Knudson [1971] and Knudson et al. [1975] proposed a two-stage model for cancer initiation to describe the incidence of both sporadic and hereditary retinoblastomas. According to Knudson's model, a tissue cell is transformed into a tumor cell after sustaining two irreversible mutations. This is often referred to as a "2-hit" model, where the term "hit" refers to mutations in a cell. The first of these mutations is assumed to occur in one of two ways: in hereditary cases, individuals inherit the first mutation; in sporadic cases the first mutation occurs by chance in a somatic cell progenitor of the tumor. The second mutation is assumed to occur by chance in a tumor cell progenitor in both hereditary and sporadic cases.

Subsequent molecular genetic studies demonstrated that Knudson's model was correct in that both alleles of the *RB1* locus are almost always mutated in retinoblastoma [Knudson, 1996]. Moreover, one mutant *RB1* allele is inherited and the second allele is lost somatically in tumors in patients with hereditary retinoblastoma, whereas both alleles are lost somatically in sporadic retinoblastomas, as predicted [Knudson, 1996]. Many other hereditary tumors, including those associated with Li-Fraumeni syndrome [Evans and Lozano, 1997], hereditary breast and ovarian cancer syndrome [Hofmann and Schlag, 2000], hereditary adenomatous polyposis [Aaltonen, 2000] and neurofibromatosis 1 [Parada, 2000] have been shown to involve a similar "2-hit" mechanism. Functional or actual loss of both alleles of these and other tumor

supressor genes is now known to be an important pathogenic mechanism in most neoplasms [Fearon, 2001].

However, it is clear that the pathogenesis of many tumors involves more than just two 'hits'. Tumors often exhibit mutations or functional alteration of several different genetic loci, and none of these changes may be sufficient by itself to produce a malignant phenotype [Strachan and Read, 1999]. It is, therefore, of some interest to consider more complex epidemiological models than that originally formulated by Knudson. Moolgavkar and Venzon [1979] introduced an alternate mathematical formulation of Knudson's 2-hit model that incorporated both the growth and death of cells in the tissue at risk; this model is depicted in Figure 1. This formulation is also convenient to extend to a 3-hit model; such a model (Figure 2) would require that a tissue cell sustain three mutations to develop into a tumor cell.

Neurofibromatosis 2 (NF2) is a dominantly-inherited tumor predisposition syndrome [MacCollin, 1999; MacCollin and Stemmer-Rachaminov, 1999]. Most affected patients develop bilateral vestibular schwannomas (VS), and many develop schwannomas of other nerves, meningiomas, ependymomas, and/or astrocytomas. Mutation of both alleles of the *NF2* locus have been demonstrated in tumors from patients with NF2 as well as in sporadic VS in patients who do not have NF2 [Evans et al., 2000]. In accordance with the Knudson hypothesis, a germ-line *NF2* mutation can be demonstrated in most, and is presumed to exist in all, patients with NF2 [Evans et al., 2000].

The purpose of this study is to fit a selection of multistage models for tumor cell development to data from patients with NF2. VS are tumors of the Schwann cells that surround the vestibular nerves; hence the tissue at risk for our study is the pool of Schwann cells that surround both vestibular nerves. We will examine the fit of two different 2-hit models and a 3-hit model to our patient data.

MODELS AND METHODS

Description of the Data

The patient data used to fit the models were obtained from the Manchester NF2 database, which contains clinical and genetic information on a large number of NF2 patients ascertained through medical specialists throughout the United Kingdom [Baser et al., 2002]. Information available for each patient included the age at onset of the first VS, whether the VS was unilateral or bilateral, and family history. The database contains 163 NF2 probands who had sufficient information for inclusion in this study. For the analyses that follow, we assume that this sample is representative of all NF2 patients. The dependent variable that we model is the age at occurrence of the first tumor cell. We assume that the time between development of the first tumor cell and the age at which the first VS is detected is roughly constant (the latter does not affect model comparisons).

Moolgavkar and Venzon's 2-hit Model

The two-mutation model for hereditary tumors presented by Moolgavkar and Venzon [1979] is appropriate for NF2 patients who have one mutant copy of the *NF2* gene present in all cells at birth. According to this model, a Schwann cell in an NF2 patient becomes a tumor cell when a second mutation occurs, inactivating the normal *NF2* gene. This model assumes that in a small interval of time, Δt, a Schwann cell divides into two Schwann cells with probability $\alpha\Delta t + o(\Delta t)$; dies with probability $\beta\Delta t + o(\Delta t)$; or mutates to form a tumor cell with probability $\mu\Delta t + o(\Delta t)$. The probability that more than one event occurs in this interval of time is $o(\Delta t)$. We will use the notation $\boldsymbol{\theta}$ to denote the vector of model parameters (α, β, μ). Additionally, it is assumed that cells behave independently of one another and that mutations occur during cell division.

We will refer to two fundamental statistical quantities throughout this paper; namely the hazard function and the probability distribution function. Let $h(t|\boldsymbol{\theta})$ denote the hazard

function for the random variable T; here $\boldsymbol{\theta}$ is used to denote the vector of parameters from a parametric model for T. This function represents the instantaneous risk of tumor at time t in a previously tumor-free tissue and is defined in the following fashion:

$$h(t|\boldsymbol{\theta}) = \lim_{\Delta t \to 0} \frac{\Pr(t \leq T < t + \Delta t \mid T \geq t; \boldsymbol{\theta})}{\Delta t}. \tag{1}$$

The probability distribution function for the random variable T, denoted by $F(t|\boldsymbol{\theta})$, represents the probability that an individual will develop a tumor prior to, or at, time t. We can express $F(t|\boldsymbol{\theta})$ in terms of the hazard function given above:

$$F(t|\boldsymbol{\theta}) = 1 - \exp\Big\{-\int_0^t h(u|\boldsymbol{\theta})du\Big\}. \tag{2}$$

It is worth noting that the probability density function for T is given by $f(t|\boldsymbol{\theta}) = F'(t|\boldsymbol{\theta}) = h(t|\boldsymbol{\theta})\{1 - F(t|\boldsymbol{\theta})\}$.

For this model, Moolgavkar and Venzon [1979] showed that the hazard function for the random variable T, representing the age at occurrence of the somatic mutation (i.e., of the first tumor cell), is given by:

$$h(t|\boldsymbol{\theta}) \;=\; -\alpha\phi(1,0,t) + (\alpha + \beta + \mu) - \beta(\phi(1,0,t))^{-1},$$

where

$$\phi(1,0,t) = \frac{C_1 - C_2\left[\frac{1-C_1}{1-C_2}\exp\{\alpha(C_1 - C_2)t\}\right]}{1 - \left[\frac{1-C_1}{1-C_2}\exp\{\alpha(C_1 - C_2)t\}\right]}. \tag{3}$$

and

$$\begin{aligned} C_1 &= \frac{1}{2\alpha}(\alpha + \beta + \mu) - \frac{1}{2\alpha}\sqrt{\alpha^2 - 2\alpha\beta + 2\alpha\mu + \beta^2 + 2\beta\mu + \mu^2}, \\ C_2 &= \frac{1}{2\alpha}(\alpha + \beta + \mu) + \frac{1}{2\alpha}\sqrt{\alpha^2 - 2\alpha\beta + 2\alpha\mu + \beta^2 + 2\beta\mu + \mu^2}. \end{aligned} \tag{4}$$

Note that $h(t|\boldsymbol{\theta})$ is the hazard function for T assuming a single initial tissue cell. If we assume that the tissue contains N cells initially, then the hazard function for the entire tissue, $h_N(t|\boldsymbol{\theta})$, is simply $Nh(t|\boldsymbol{\theta})$; this follows directly from the assumption that the cells

behave independently of one another. A likelihood for the data can be constructed in the customary fashion [Lawless, 1982] with patient i, having tumor onset time t_i, contributing $h_N(t_i|\boldsymbol{\theta})\{1-F_N(t_i|\boldsymbol{\theta})\}$ to the likelihood. The likelihood, as a function of $\boldsymbol{\theta}$, can be maximized by means of a quasi-Newton algorithm [Nash, 1990] to obtain maximum likelihood estimates (MLE) of the model parameters.

Nonhomogeneous Poisson Process

Another approach to modelling this two-mutation process is to make some additional assumptions regarding the division and death of the tissue cells. If the tissue were assumed to grow according to a deterministic process, then the number of cells present in the tissue at any time t can be given by a function $X(t)$. If a reasonable functional form for $X(t)$ could be chosen and it is assumed that there is a small chance that any of the tissue cells mutate, then the generation of tumor cells can be modelled according to a nonhomogeneous Poisson process. If we assume that the rate at which tissue cells mutate is a constant μ, then the intensity of the process is simply $h(t|\mu) = \mu X(t)$; which is of course the hazard function for the age at occurrence of the somatic mutation. We will refer to this model as the 'Poisson' model.

A likelihood for the data is easily constructed for this model, and the MLE for the mutation rate has a simple, closed form solution. Again, the contribution of patient i to the likelihood function is $f(t_i|\mu) = h(t_i|\mu)\{1 - F(t_i|\mu)\}$, and the MLE for the mutation rate, estimated from a sample of n patients, is given by:

$$\hat{\mu} = \frac{n}{\sum_{i=1}^{n} \left(\int_0^{t_i} X(s)ds \right)}. \tag{5}$$

It is important to note that the choice of the function $X(t)$ clearly influences the estimate of the mutation rate. The integral in the expression for the estimate of the mutation rate may require numerical integration if the function chosen for $X(t)$ is not conveniently integrable.

3-hit Model

Thus far we have assumed that tumorigenesis is a two-mutation process and that a tumor cell is generated when a normal Schwann cell sustains two irreversible mutations. Here we explore a model that assumes that a tumor cell is the end result of a normal tissue cell sustaining three irreversible mutations; this model will be referred to as the '3-hit' model. In NF2 patients, the first of these mutations has already been sustained prior to birth and the two subsequent mutations are assumed to occur by chance in the somatic tissue. We assume for simplicity that these two mutations occur at a constant, common mutation rate μ. Moolgavkar and Luebeck [1990] discuss a model that is appropriate for this process.

Again we denote the number of Schwann cells in our tissue of interest by $X(t)$; recall that these cells have already sustained a single mutation. As in the case of the Poisson model we must specify a functional form for $X(t)$ to fit our model to patient data. We will refer to cells that have sustained a second but not a third mutation as intermediate cells, and cells that have sustained three mutations as tumor cells. In a small interval of time Δt, the probability that an intermediate cell is generated by the mutation of a tissue cell is $\mu X(t)\Delta t + o(\Delta t)$. The probability that more than one intermediate cell is generated in this fashion is $o(\Delta t)$. The growth, death and mutation of the intermediate cells are assumed to follow a birth-death process. Again, in a small interval of time Δt, an intermediate cell divides into two intermediate cells with probability $\alpha\Delta t + o(\Delta t)$, dies with probability $\beta\Delta t + o(\Delta t)$, and divides into an intermediate cell and a tumor cell with probability $\mu\Delta t + o(\Delta t)$. The probability of more than one such event occurring in this time interval is $o(\Delta t)$. Moolgavkar and Luebeck showed that for such a model the hazard function is given by:

$$h(t|\boldsymbol{\theta}) = \mu^2 \int_0^t X(s) \exp\{g(\boldsymbol{\theta}; t-s)\} ds, \tag{6}$$

where

$$\begin{aligned} g(\boldsymbol{\theta}; t-s) &= 2\alpha C_1 (t-s) + 2\log\left\{ \frac{1 - \frac{1-C_1}{1-C_2}}{1 - \frac{1-C_1}{1-C_2} \exp\{\alpha(C_1 - C_2)(t-s)\}} \right\} \\ &- (\alpha + \beta + \mu)(t-s), \end{aligned} \tag{7}$$

and C_1 and C_2 are defined as above.

A likelihood for the data, as a function of $\boldsymbol{\theta}$, can be constructed in an identical fashion to the previous models, and estimates for the model parameters can be found by numerically maximizing the likelihood. The integral in (6) can be calculated numerically.

RESULTS

In our fitting of the 2-hit model, we have assumed that there are 20 cells initially in the tissue that gives rise to a vestibular schwannoma, i.e., $N = 20$ for the Schwann cell precursors of a vestibular nerve. The actual number of initial cells from which the Schwann cells surround a vestibular nerve is unknown. $N = 20$ was chosen as a reasonable guess, but as discussed below, the precise value used is not crucial. The MLEs for the model parameters are presented with their estimated standard errors in Table I; the value of the log-likelihood evaluated at the MLE is also provided. The estimated model parameters can also be input into the expression for the distribution function $F_N(t|\boldsymbol{\theta})$ and plotted as a function of age. This permits a comparison between the model-estimated distribution function and the empirical distribution function for the data.

The estimated and empirical distribution functions are plotted in Figure 3. The overall model fit is reasonable; however the model-estimated incidence of tumor is much higher than the observed incidence for ages less than 20 years.

To enable the fitting of both the Poisson and 3-hit models to patient data, we must include an estimate of the number of Schwann cells in the tissue as a function of age. This is based on the theory on the growth of the Schwann cells outlined in the Appendix, and is given below:

$$X(t) = \begin{cases} \exp\left\{\log(20) + \frac{\log(5550)t}{0.46}\right\}, & t < 0.46, \\ 111000, & t \geq 0.46. \end{cases} \tag{8}$$

This function asymptotes to a value of 111000 and has exponential growth for time $t < 0.46$ years [24 weeks]. Note that time for this function means time since conception and not

Table I: Summary of parameter estimates from model fitting

Model: 2-hit Model		
Value of log-likelihood: -660.0		
Akaike information criterion: -663.0		
Parameter	Estimate	SE
α	4.214×10^{-1}	1.010×10^{-1}
β	3.352×10^{-1}	1.013×10^{-1}
μ	3.154×10^{-4}	0.642×10^{-4}

Model: Poisson Model		
Value of log-likelihood: -717.2		
Akaike information criterion: -718.2		
Parameter	Estimate	SE
μ	3.007×10^{-7}	0.235×10^{-7}

Model: 3-hit Model		
Value of log-likelihood: -651.2		
Akaike information criterion: -654.2		
Parameter	Estimate	SE
α	8.276×10^{-1}	0.067×10^{-1}
β	8.108×10^{-1}	0.084×10^{-1}
μ	1.159×10^{-4}	0.096×10^{-4}

birth. Integration of this function is required in the likelihoods for both the Poisson and 3-hit models and has been performed numerically with Romberg integration [Mathews, 1992].

The estimate of the mutation rate from the Poisson model, its estimated standard error, and the value of the log-likelihood evaluated at the estimate are presented in Table I. The Poisson model-estimated distribution function for the age at onset of the first tumor cell is plotted in Figure 3 to allow for comparison with the empirical distribution function and the 2-hit model. The fit of the Poisson model to the data is quite poor.

A similar display of model parameter estimates is provided in Table I for the 3-hit model. The model-estimated distribution function for the 3-hit model is also included in Figure 3. The model-estimated distribution function fits the data very closely for the 3-hit model, except for ages less than 18.

An additional method of comparing the fit of the models to the data is to use the Akaike

Information Criterion [Sakamoto et al., 1986], which adds a penalty term for the number of model parameters to the log-likelihood. The ordering of the three models according to this criterion is consistent with the quality of model fit observed in Figure 3: the 3-hit model provides the best fit to the data, the 2-hit model provides the next best fit, and the Poisson model provides the poorest fit of the three considered.

DISCUSSION

The models we have described here all make assumptions about the growth of the tissue at risk for developing the tumor in some way; here we discuss the sensitivity of our results to these assumptions. In the Poisson and 3-hit models, we have estimated the number of Schwann cells in the tissue as a function of age, based on the theory given in the Appendix. Small perturbations of this assumed growth curve did not significantly affect the final fitted distribution functions. The 2-hit model also required us to specify a constant representing the number of Schwann cell precursors in the vestibular nerve; we have chosen $N = 20$. To explore the sensitivity of our results to the choice of this constant, we fit the 2-hit model several times assuming a range of values for the N constant; we tried several values between 10 and 200. The model-estimated distribution functions produced from these fittings were identical to one another. Changing the N constant only affects the estimates of the model parameters as they become rescaled when the value of N is perturbed. The effect on the mutation rate was quite small, providing estimates ranging from 3.52×10^{-5} to 6.20×10^{-4}. Although the raw values of the cell growth and death rate parameter estimates are affected by changes to the value of N selected, the difference between these estimates remains constant.

The Poisson model provides a poor fit to the data, and the 2-hit and 3-hit model fit the model well except for ages less than 18 years. The 3-hit model provides a marginally better fit according to Figure 3. The result for the Poisson model is not surprising, as it estimates only a single parameter from the data. Thus the Poisson model is much less

flexible than the other two models. The slightly superior fit of the 3-hit model (based on Akaike information criterion) may suggest that the pathogenesis of these tumors requires more than the two mutations of a Schwann cell. Other tumors, most notably colon cancer, have been shown to develop after a multistep pathogenesis. Moolgavkar and Luebeck [1992] have used similar models to those described here to model the multistep pathogenesis of colon cancer accurately. Other authors have also discussed the pathogenesis of colon cancer as a multistage process [Chung, 2000; Kinzler and Vogelstein, 1996]. The notion that more than two mutations are required to produce a schwannoma in NF2 is supported by molecular genetic studies [Lamszus et al., 2000; Bruder et al., 1999]. There is clearly a need for further molecular genetic studies to examine the effect of other genes on the development of NF2-associated tumors.

The methodology discussed here can also be applied to patient data for other NF2-tumors, such as meningiomas and epidymomas. As well, it would be interesting to examine the effect of adding genotype information into these models. Genotype-phenotype correlations have been observed in NF2, and the inclusion of a patient's genotype in the model may result in a model that fits the data more closely.

APPENDIX: Neuroanatomy of the human vestibular nerve

Axons are present in the vestibular nerve by the third week of human fetal development [Cooper, 1948]. Schwann cell myelin is visible at the light microscopic level in the peripheral VIIIth nerve (which includes the vestibular nerve) by the 22th fetal week; the first thin myelin sheaths are not visible at a light microscopic level, but the entire process of Schwann cell attachment to the VIIIth nerve may occur within 1–3 weeks [J Moore and FH Linthicum, Jr., personal communication]. Oligodendrocyte myelin is visible in the central vestibular nerve by the 26th fetal week [Moore et al., 1995]. In newly-myelinated axons, all internodal

segments of myelin are approximately the same length [Hiscoe, 1947; Vizoso, 1950]. As nerves lengthen due to body growth, no new internodal segments of myelin are added, but those that are already present lengthen [Vizoso, 1950]. As a result, the number of Schwann cells remains constant.

Since our model is based on the process of mutation in dividing Schwann cells (i.e., during fetal growth), an estimate is needed of the number of Schwann cells in the human fetal vestibular nerve. This can be estimated directly as the product of (a) the number of neurons in the vestibular nerve, (b) the length of the myelinated vestibular nerve axons, and (c) the distance between nodes of Ranvier in the myelin sheath (internode distance). An indirect estimate is necessary because there are no published data for any of these variables for the human fetal vestibular nerve. Since the number of Schwann cells remains constant during post-natal growth, data from adults can be used to provide an indirect estimate of the number of Schwann cells in the fetal vestibular nerve:

(a) The number of neurons in individual human vestibular nerves in people aged < 50 years ranges from about 14,000 to about 22,000, with a mean of about 18,500 [Rasmussen, 1940; Naufal and Schuknecht, 1972; Bergström 1973a; Richter, 1980]. The number of neurons in the vestibular nerve decreases after about age 50 [Bergström, 1973a; Rosenhall, 1973; Richter, 1980].

(b) The maximum length of myelinated vestibular nerve axons is most appropriately measured by the distance between the cribriform area and the Schwann cell-glial junction. In three temporal bone specimens, the distances were 5.1 mm (57 year old female), 7.0 mm (75 year old male), and 6.9 mm (90 year old male), an average of about 6.0 mm [FH Linthicum, Jr., personal communication].

(c) Vizoso [1950] reported that internode length increased with growth in human nerves. For example, from a presumed 230 μm at birth, the internodes in the facial nerve of an 18 year old female were 550 μm. In addition, there are data on the diameter of vestibular

nerve axons, and on the relationship between fiber diameter and internode distance in other nerves. Vestibular nerve axon diameters range from 7–15 μm [Natout et al., 1987; distribution not given]. In human ventral sacral ventral nerve roots, the relationship between fiber diameter and internode distance is about $l/d = 100$, where l = internode length (in μm) and d = fiber diameter (in μm) [Schalow, 1989]. Extrapolating to the vestibular nerve, axons 10 μm in diameter would have internodes 1.0 mm in length. A reasonable range for the mean internode length in the vestibular nerve could be about 0.5–1.0 mm.

Therefore, an indirect point estimate for the number of Schwann cells in the human fetal vestibular nerve is 18,500 fibers/nerve $\times$ 6.0 mm/axon $\times$ 0.5 mm/internode $= 0.555 \times 10^5$, or 1.11×10^5 using an internode length of 1.0 mm. The corresponding totals for both vestibular nerves would be double these numbers.

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Figure 1: 2-hit model for development of vestibular schwannomas in NF2 patients. All cells in the body have at least one mutation, inherited through the germ line. Circles are used to denote cells in a given stage of the model and arrows denote the possible transitions between stages of the model; α, β and μ represent cell growth, death and mutation rates, respectively.

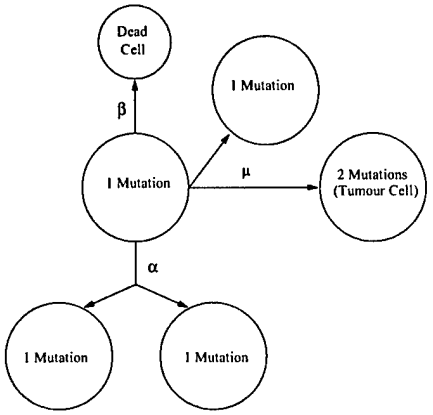


Figure 2: 3-hit model for development of vestibular schwannomas in NF2 patients. All cells in the body have at least one mutation, inherited through the germ line. Circles are used to denote cells in a given stage of the model and arrows denote the possible transitions between stages of the model; α, β and μ represent cell growth, death and mutation rates, respectively.

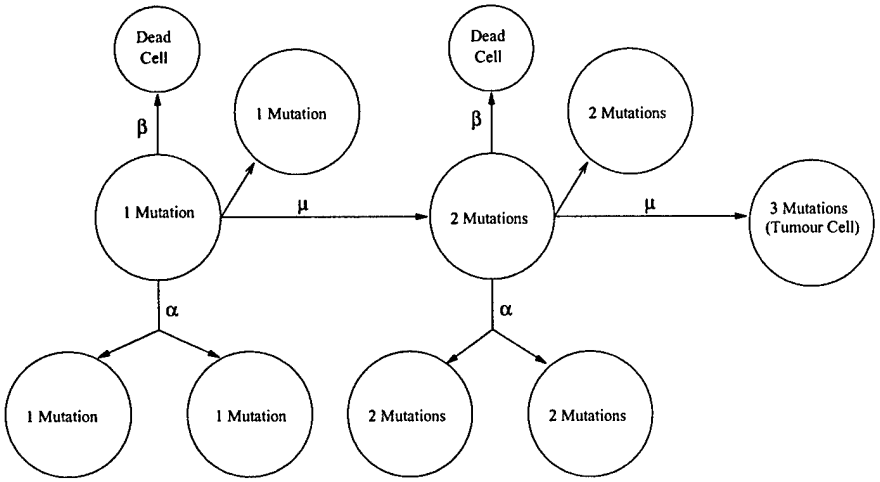
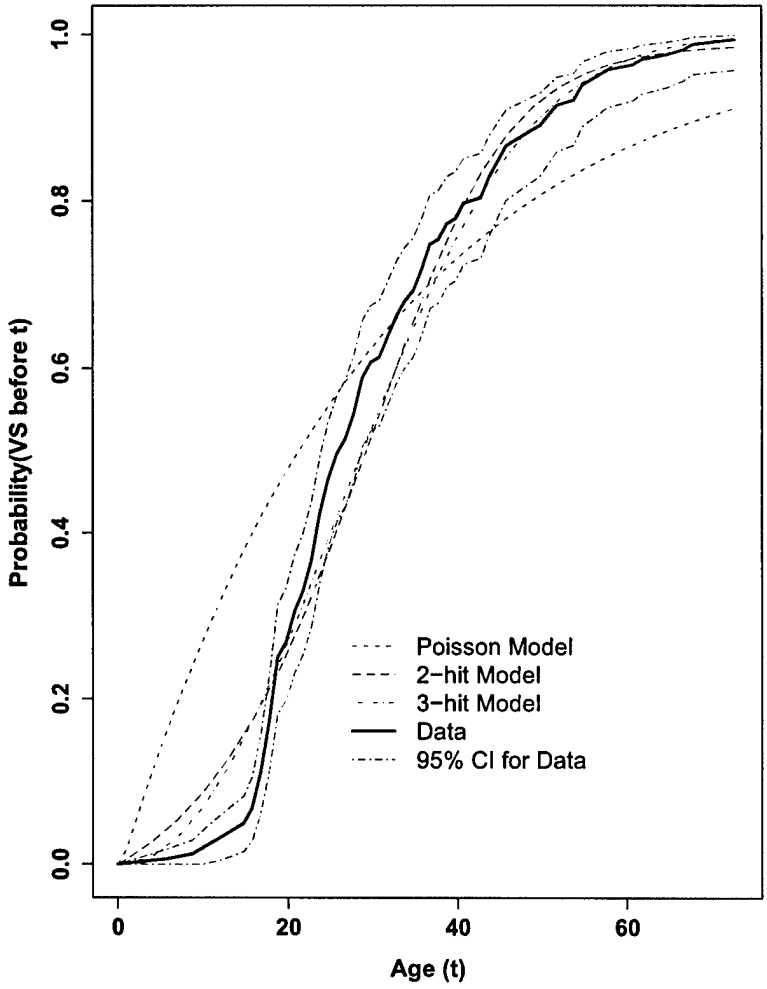


Figure 3: Model-fitted distribution functions from three models and empirical distribution function for the data.



Legends for figures

Figure 1: 2-hit model for development of vestibular schwannomas in NF2 patients. All cells in the body have at least one mutation, inherited through the germ line. Circles are used to denote cells in a given stage of the model and arrows denote the possible transitions between stages of the model; α, β and μ represent cell growth, death and mutation rates, respectively.

Figure 2: 3-hit model for development of vestibular schwannomas in NF2 patients. All cells in the body have at least one mutation, inherited through the germ line. Circles are used to denote cells in a given stage of the model and arrows denote the possible transitions between stages of the model; α, β and μ represent cell growth, death and mutation rates, respectively.

Figure 3: Model-fitted distribution functions from three models and empirical distribution function for the data.

ANALYSIS OF INTRA-FAMILIAL PHENOTYPIC VARIATION IN NEUROFIBROMATOSIS 1 (NF1)

J. Szudek[1,3], H. Joe[2] and J.M. Friedman[1]

[1]Department of Medical Genetics and [2]Department of Statistics, The University of British Columbia, Vancouver, CANADA; [3] Dalhousie Medical School, Halifax, Nova Scotia, CANADA

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Address for Correspondence:

J. M. Friedman, MD, PhD

Department of Medical Genetics

University of British Columbia

6174 University Boulevard

Vancouver, BC Canada V6T 1Z3

Tel: (604) 822-0367 Fax: (604) 822-5348

e-mail: frid@interchange.ubc.ca

ABSTRACT

The relationship of genetic factors to variable expressivity in neurofibromatosis 1 (NF1) is poorly understood. We examined familial aggregation of NF1 features among different classes of affected relatives. Clinical information was obtained from the National NF Foundation International Database on 904 affected individuals in 373 families with 2 or more members with NF1. We used multivariate probit regression to measure the associations between various classes of relatives for each of 10 clinical features of NF1 while simultaneously adjusting for covariates including related features, age and gender.

Two distinct patterns were observed when we compared associations between first and second-degree relatives, sibs, and parent-child pairs: Lisch nodules and café-au-lait spots had greater associations between first-degree relatives than between second-degree relatives, while subcutaneous neurofibromas, plexiform neurofibromas, café-au-lait spots, and intertriginous freckling had greater associations between sibs than between parents and children. In addition, Lisch nodules, subcutaneous neurofibromas and cutaneous neurofibromas had greater associations between affected fathers and children than between affected mothers and children. These familial patterns suggest that unlinked modifying genes and the normal NF1 allele may both be involved in the development of particular clinical features of NF1, but that the relative contributions vary for different features.

Key Words: Neurofibromatosis 1, familial correlation, multivariate probit regression

INTRODUCTION

Neurofibromatosis 1 (NF1) is an autosomal dominant disease that affects about 1/3,500 people [Friedman, 1999]. NF1 can affect the skin, skeleton and nervous system and is characterised by highly variable expressivity [Friedman *et al.*, 1999]. Many disease features are progressive, but the rate of progression and the occurrence of serious manifestations vary greatly from one patient to another [Friedman and Riccardi, 1999]. This variability and the confounding effect of age have hindered efforts to characterise the relationship of genetic factors at the *NF1* locus or other loci to disease variability.

More than 400 different constitutional *NF1* mutations have been reported [Fahsold *et al.*, 2000; Korf, 1999; Messiaen *et al.*, 2000]. In general, little evidence has been found of allele-phenotype correlations in NF1, although a more or less consistent phenotype occurs in association with deletions involving the entire *NF1* gene [Dorschner *et al.*, 2000; Tonsgard *et al.*, 1997]. Similar clinical features have been observed among affected members of a few families with the NF1 variants Watson syndrome [Allanson *et al.*, 1991], familial café-au-lait spots [Abeliovich *et al.*, 1995] or familial spinal neurofibromas [Ars *et al.*, 1998; Poyhonen *et al.*, 1997; Pulst *et al.*, 1991]. This observation is consistent with an allele-phenotype correlation, but no consistent kind of *NF1* mutation has been found in families with these or other phenotypic variants. Affected members of a single family with typical NF1 often have quite different disease phenotypes, despite sharing an identical mutant *NF1* allele. Clearly, variation in the mutant *NF1* allele itself does not account for all of the variability seen in most disease features.

Easton et al. [Easton *et al.*, 1993] studied the expressivity of NF1 in 175 affected members of 48 families and found statistically significant correlations for the number of café-au-lait spots, the number of dermal discrete neurofibromas and head circumference among affected relatives.

Comparison of the strength of these correlations in relatives of different classes provided evidence for modifying genes influencing the number of café-au-lait spots.

We have shown that several statistically significant associations exist between the occurrence of individual clinical features in 3067 unrelated probands with NF1 [Szudek *et al.*, 2000b; Szudek *et al.*, (Submitted for publication)]. We also found significant associations in the occurrence of Lisch nodules, optic glioma, learning disability, macrocephaly and short stature in affected parent-child pairs [Szudek *et al.*, 2000b] but made no attempt to adjust for the non-independence of multiple relative-pairs from the same family or for associations among clinical features in individuals in this preliminary study. We now extend our analysis to measure correlations of NF1 features among relatives of various classes using methods that take other clinical features, gender and age into account and adjust for the non-independence of affected relatives. By comparing the correlations observed, we provide evidence that genetic sources of variation are generally important in NF1 and vary for different clinical features.

SUBJECTS AND METHODS

Subjects

All patients in this study met the NIH diagnostic criteria for NF1 [Gutmann *et al.*, 1997; NIH, 1988]. Data were obtained from the National NF Foundation International Database (NFDB) [Friedman *et al.*, 1993]. The NFDB contains extensive demographic, clinical, and genetic data on NF1 patients from more than 20 participating clinical centres in North America, Europe, Japan, and Australia. All information is recorded using a standard format and consistent definitions of clinical features. The greatest strength of the NFDB is its large size. The

limitations include the fact that data are contributed by staff members of specialized neurofibromatosis clinics, which probably produces an ascertainment bias.

At the time of this study, the available dataset included 373 families with two or more affected members, for a total of 904 individuals with NF1. 346 of these were nuclear families that included either an affected parent and one or more affected children or two or more affected sibs. 27 families were more extended, including a total of 74 second-degree affected relative pairs. The family sizes ranged from 2 to 7 affected members: There were 272 families with two affected members, 65 with three, 24 with four, 6 with five, 3 with 6, and 3 with 7 affected members included in the study.

For analysis of familiality, we selected 10 clinical features of NF1: café-au-lait spots, intertriginous freckling, Lisch nodules, cutaneous neurofibromas, subcutaneous neurofibromas, plexiform neurofibromas, seizures, scoliosis, optic glioma and neoplasms other than neurofibromas or optic gliomas ("other neoplasms"). Most of these features were identified by physical examination, and all were treated as binary variables. Café-au-lait spots were coded as "present" if the subject had 6 or more spots. Cutaneous or subcutaneous neurofibromas were coded as "present" if the subject had two or more lesions of the same type. Plexiform neurofibroma was coded as "present" if the subject had one or more lesions. Lisch nodules were diagnosed or excluded by slit lamp examination. The presence or absence of optic glioma was determined by cranial MRI or CT examination. Only patients with definite presence or absence of a feature were considered in models involving that feature. The complete data set used in this study is available from the authors by request.

Statistical Methods

Familial correlations in each class of relatives were estimated for clinical features measured as binary (presence/absence) variables by means of a multivariate probit model [Ashford and Sowden, 1970; Joe, 1995; Mendell and Elston, 1974]. For a particular binary response variable, the covariates used for adjustment were chosen on the basis of a univariate probit or logistic stepwise regression, ignoring the familial dependence. These covariates were then used in the multivariate probit model. We included age as a covariate in all analyses because many NF1 features have a higher prevalence in older patients [DeBella *et al.*, 2000]. We have shown previously that clinical features do not occur independently in NF1 patients, even after adjusting for the effect of age [Szudek *et al.*, 2000b; Szudek *et al.*, (Submitted for publication)]. Therefore, we also included the binary variables representing presence or absence of other associated features, as well as gender, as covariates to minimize confounding. In addition, we considered stature and head circumference as covariates after standardizing the measurements for each patient to age- and gender-specific population norms [Szudek et al., 2000a].

For the multivariate probit model, the binary response vector is $(Y_1,...,Y_k)$ for a family of size k, Y_j is 1 if a latent variable $Z_j \leq \beta_0 + \beta'x$, where β_0 is an intercept and β is a vector of regression coefficients for the covariate vector x. $(Z_1,...,Z_k)$ have a joint multivariate normal distribution with zero mean vector and correlation matrix R, where the (latent) correlation for a given pair depends on the relation type of the pair.

The estimates of the regression coefficients and latent correlation coefficients, together with an estimated covariance matrix and standard errors, were obtained by numerical maximum likelihood, using the quasi-Newton algorithm [Nash, 1990]. The multivariate normal rectangle probabilities for the multivariate probit model were computed using fast approximation methods [Joe, 1995]; the first order approximation requires only bivariate normal rectangle probabilities, and the second order approximation requires multivariate probabilities up to the fourth dimension. These approximations have made feasible the computations for the multivariate probit model; they are much more accurate than older methods such as the approximation of Mendell and Elston [Mendell and Elston, 1974].

Univariate probit regressions were used to obtain appropriate functions for age (e.g., $e^{-age/4}$) and initial estimates of regression coefficients for covariates representing related features, interactions between related features, and gender. Familial aggregation was assessed among sibs, parent-child pairs (including mother-child and father-child pairs separately) and second-degree relatives with a multivariate probit model.

Parameters and coefficients with 95% confidence intervals that excluded zero were deemed statistically significant. Standard errors and covariance matrices were used to test for differences between intra-familial correlation coefficients for different comparisons. For example, to test for a difference between sib-sib correlation and parent-child correlation we used the following formula:

$$Z = \frac{r_{ss} - r_{pc}}{s} \quad \text{where} \quad s = \sqrt{(SE_{rss})^2 + (SE_{rpc})^2 - 2\,\mathrm{cov}(r_{ss}, r_{pc})}$$

Z-scores were converted into p-values according to the standard normal distribution. We used one-tailed tests to compare correlations between first-degree and second-degree relatives and between sib pairs and parent-child pairs because we had a prior expectation that correlations

between sibs would be at least as strong as those between parents and children [Easton *et al.*, 1993; Szudek *et al.*, 2000b]. We used two-tailed tests to compare mother-child correlations to father-child correlations.

RESULTS

We studied 904 individuals with NF1 from 373 families with two or more affected members. 91% of the individuals studied were White, 2% Asian, 1% Black, 1% Latin, and the remainder either of "other" or "unknown" ethnic origin. Table I shows the prevalences of each of the 10 NF1 clinical features in affected fathers, mothers and their affected children in the NFDB study sample and compares them to the prevalences in the sample used by Easton *et al.* [1993].

Familial aggregation among various classes of relatives was estimated using multivariate regression models. Table II shows the regression parameters and standard errors for the terms that were included in each model. The strength of association between the modelled feature and a covariate is measured by β. A unit increase in the value of the covariate means the modelled feature is $\exp(2\beta)$ times more likely to be present. For example, subjects with intertriginous freckling were $\exp(2\times0.52)=2.8$ times more likely also to have café-au-lait spots than subjects of the same age and gender without intertriginous freckling. Also, subjects with intertriginous freckling *and* subcutaneous neurofibromas were $\exp(2\times(0.52-.18+0.62))=6.8$ times more likely also to have café-au-lait spots.

The parameter estimates for age were highly significant ($p<0.001$) for Lisch nodules, subcutaneous neurofibromas, cutaneous neurofibromas, and intertriginous freckling; significant ($p<0.05$) for café-au-lait spots, optic gliomas and plexiform neurofibromas; and not significant

($p>0.05$) for other neoplasms, seizures or scoliosis. The parameter estimate for gender was not significant in any of the models.

Table III shows the number of sib, parent-child (including mother-child and father child) and second-degree relative pairs used in each model. Subjects were included in a model only if the status ("presence" or "absence") of the modelled feature and all covariates was known.

Figure 1 shows the adjusted intrafamilial correlation coefficients and 95% confidence intervals for 6 clinical features among 746 affected first-degree relatives and among 148 affected second-degree relatives. The multivariate probit regression failed to converge on correlation coefficients between second-degree relatives for optic glioma, other neoplasms, seizures or scoliosis because of the low frequency of these features and insufficient sample size. We did obtain correlation coefficients between first-degree relatives for these features, but none of these correlations was significantly different from zero. Statistically significant positive correlations between first-degree relatives were found for 5 of the 6 other features listed in Figure 1. Significant positive correlations between second-degree relatives were also found for 2 of these 6 features. Significant negative correlations were not observed for any of the features. Correlations were significantly greater among first-degree relatives than among second-degree relatives for Lisch nodules ($p=0.0001$) and café-au-lait spots ($p=0.0004$). Correlations among first-degree relatives were not statistically different from correlations among second-degree relatives for subcutaneous neurofibromas ($p=0.06$), cutaneous neurofibromas ($p=0.49$), intertriginous freckling ($p=0.07$) or plexiform neurofibromas ($p=0.11$).

Figure 2 shows the adjusted intrafamilial correlation coefficients and 95% confidence intervals for 6 features among 268 affected sib pairs and among 373 affected parent-child pairs. Again, the multivariate probit regression failed to converge on correlation coefficients between

sibs or parent-child pairs for optic glioma, other neoplasms, seizures or scoliosis. Statistically significant positive correlations between sibs were found for all 6 features in Figure 2. Significant positive correlations between parents and children were found for 4 of the 6 features. Significant negative correlations were not observed for any of the features. Correlations were significantly greater between sibs than between parents and children for subcutaneous neurofibromas ($p=0.04$), café-au-lait spots ($p=0.001$), intertriginous freckling ($p=0.03$) and plexiform neurofibromas ($p=0.02$). Correlations between sibs were not statistically different from the correlations between parents and children for Lisch nodules ($p=0.40$) or cutaneous neurofibromas ($p=0.29$).

Figure 3 shows the adjusted intrafamilial correlation coefficients and 95% confidence intervals for 6 features between 233 affected mother-child pairs and between 140 affected father-child pairs. Statistically significant positive correlations between mothers and children were found for 3 of the 6 features. Significant positive correlations between fathers and children were found for 4 of the 6 features. Significant negative correlations were not observed for any of the features in either relationship. Correlations between fathers and children are significantly greater than correlations between mothers and children for Lisch nodules ($p=0.001$), subcutaneous neurofibromas ($p=0.0001$) and cutaneous neurofibromas ($p=0.02$). Correlations do not differ significantly between father-child pairs and mother-child pairs for café-au-lait spots ($p=0.62$), intertriginous freckling ($p=0.71$) or plexiform neurofibromas ($p=0.17$).

DISCUSSION

Limitations of Our Data and Methods

We analysed familial latent correlations for 10 NF1 clinical features while adjusting for other related features, age and gender through statistical modelling. We were able to test for differences between correlations among various classes of relatives for 6 of the 10 features studied. Differences between various classes of relatives were found for each of these 6 features (Figures 1-3).

The NFDB draws its information from specialised clinics, so we were concerned about the representativeness of our sample. However, frequencies of features found among the familial cases used in this study (Table I) are comparable to those seen in another family study of variable NF1 expressivity [Easton *et al.*, 1993] and in two available population-based studies of NF1 patients [Huson *et al.*, 1989; Samuelsson and Axelsson, 1981].

Easton *et al.* [1993] studied 175 individuals with NF1 from 48 families, including 6 pairs of monozygotic twins, 76 pairs of sibs, 60 parent-offspring pairs, 54 second-degree relative pairs and 43 3rd degree relative pairs. These investigators examined 8 NF1 clinical features and found significant intrafamilial correlations for 3 quantitative variables: number of café-au-lait spots, number of cutaneous neurofibromas and head circumference. Easton and his associates also analysed 5 traits as binary variables, but these comparisons did not include adjustment for age. Furthermore, none of their analyses adjusted for the non-independence of multiple relative-pairs from the same family or of various clinical features. Our sample size is 5 times larger, and we examined 10 clinical features, 6 of which are the same as Easton's. Also, we included associations between features as covariates in the familial analyses. Unlike Easton et al., we did

not have counts of café-au-lait spots and dermal discrete neurofibromas, but Easton's quantitative investigations of these features complement our binary analyses nicely. Both studies found evidence of modifying genes on café-au-lait spots but not on dermal discrete neurofibromas.

All of the features we studied were treated as binary variables. Many of the clinical features of NF1 (and other diseases) are by nature binary, and ours is the first study to examine correlations for binary traits among different familial relationships while accounting for continuous covariates such as age. Similar methods have been used to study lens opacities [Anonymous, 1994] and liver cancer [Liang and Beaty, 1991] in individuals who do not have NF1, but we may be the first to study an autosomal dominant disease in this manner.

Although this is by far the largest group of NF1 families ever studied, we only had 74 pairs of second-degree relatives. Models for most features used even fewer second-degree relatives because the data were incomplete. Subjects were included in a model only if the status of the modelled feature and of all covariates was known (Table III). These relatively small sample sizes are reflected in the wide 95% confidence intervals for the correlation coefficients among second-degree relatives (Figure 1). Statistical techniques are less reliable for smaller sample sizes, so we must attach an additional note of caution to the point estimates for the correlation coefficients between second-degree relatives, particularly for Lisch nodules and intertriginous freckling, in which the analysis included only 35 pairs of second-degree relatives (Table III).

Several features had significantly positive correlations among second-degree relatives, but none of these correlations was significantly greater than that for the same feature among first-degree relatives (Figure 1). Similarly, several features had significantly positive correlations between parents and children, but none of the correlations was greater than that for the same

feature between sibs (Figure 2). The absence of significant negative correlations supports the statistical validity of our approach. One would expect to observe negative, as well as positive, correlations by chance when making multiple comparisons.

The most important confounding factor in familial analyses of NF1 is age. Many disease features are more prevalent in older NF1 patients [Cnossen *et al.*, 1998], and, if not appropriately controlled, age might produce a correlation between affected relatives of similar age (e.g., sibs) or obscure a correlation between relatives of very different ages (e.g., parents and children). Our multivariate models minimise the confounding effect of age, but they may not eliminate it completely. The covariate representing age was significant in models for most features, but it is possible that a residual age effect is contributing to the observed differences between sib-sib and parent-child pairs for features such as subcutaneous neurofibromas and intertriginous freckling that become more prevalent with age (Figure 2). Age is less likely to influence the intrafamilial correlations for café-au-lait spots or plexiform neurofibromas, which, when considered as discrete variables, occur with a relatively stable frequency with age [DeBella *et al.*, 2000; Friedman *et al.*, 1999].

Patterns of Associations of Clinical Features Among Relatives

Lisch nodules and café-au-lait spots had significantly higher correlations among first-degree relatives than among second-degree relatives. Higher correlations for first than second-degree relatives would be expected for effects produced by modifying genes at unlinked loci but might also result from environmental factors that are more likely to be shared among closer relatives. Our observations are consistent with the effect of a modifying gene on the pathogenesis of Lisch nodules.

Easton *et al.* [1993] found a higher correlation for café-au-lait spots between monozygotic twins than between sibs, suggesting the effect of a genetic locus or loci in addition to *NF1*. Our findings of a strong correlation for café-au-lait spots in first-degree relatives but no correlation among second-degree relatives are consistent with this interpretation.

Lisch nodules and café-au-lait spots share an origin from neural crest-derived tissue, but this is also true of some other lesions characteristic of NF1, including neurofibromas of all types and intertriginous freckling [Bolande, 1981]. We previously reported an association between the occurrence of Lisch nodules and café-au-lait spots in individual NF1 patients [Szudek *et al.*, 2000b], but intertriginous freckling was also associated – a feature that shows no indication of a stronger familial correlation among first-degree than second-degree relatives (Figure 1).

Intertriginous freckling, subcutaneous neurofibromas, plexiform neurofibromas and café-au-lait spots had higher correlations between sibs than between parents and children. Easton *et al.* [1993] found that concordance for dermal discrete neurofibromas (which include subcutaneous neurofibromas) between monozygotic twins was much higher than between sibs, an observation that suggests the involvement of a genetic factor. Affected sibs would be expected to share the same normal *NF1* allele by descent half of the time, but parent-child pairs rarely would. Effects

of functional polymorphisms of the normal *NF1* allele might explain a higher correlation of these features among sib pairs than among parent-child pairs. Another possible explanation is differences in environmental factors that are more likely to be shared among sibs than between a parent and child.

Intertriginous freckling, subcutaneous neurofibromas, plexiform neurofibromas and café-au-lait spots all share an origin from neural crest-derived cells. We found that café-au-lait spots and intertriginous freckling tended to occur together in individual NF1 patients, and so did cutaneous, subcutaneous, and plexiform neurofibromas, but associations were not seen between the features in these two groups [Szudek *et al.*, (Submitted for publication)]. In the present study, we did not find a stronger correlation for cutaneous neurofibromas in sibs than in parent-child pairs, as we did for subcutaneous and plexiform neurofibromas (Figure 2).

Lisch nodules, subcutaneous neurofibromas, and cutaneous neurofibromas had higher correlations between affected fathers and children than between affected mothers and children (Figure 3). Our sample included twice as many mother-child pairs as father-child pairs, so we were concerned about ascertainment bias – the possibility that only severely affected father-child pairs tend to be seen in the NF clinics that contributed data to the NNFF International Database. However, the frequencies of all features studied were similar in affected fathers and affected mothers (Table I).

Shared environment is unlikely to be the sole cause of associations between parents and children, due to large differences in age. It is also unlikely that shared environment is responsible for the difference in correlations between mother-child and father-child pairs. Likewise, a multifactorial influence with a more extreme threshold for males than for females cannot explain the observations for these features. Gender is not a significant predictive factor in

any of our models (Table II), and feature frequencies among affected children of affected fathers are similar to those among affected children of affected mothers (Table I). Parent-of-origin effects on severity of NF1 have been suggested [Hall, 1981; Miller and Hall, 1978], but most studies do not support this possibility [Huson *et al.*, 1989; Riccardi and Wald, 1987]. Our findings are consistent with a parent-of-origin effect on the strength of the parent-child correlation rather than with a more severe phenotype in affected offspring of parents of one gender when compared to affected offspring of parents of the other gender. Similar parent-child aggregation patterns have been reported for body mass index [Friedlander *et al.*, 1988] and blood pressure [Hurwich *et al.*, 1982], but they are unprecedented in NF1. We do not know of a genetic mechanism that can explain this phenomenon.

Summary and Perspective

The patterns of familial correlations shown here suggest that genetic factors involved in determining the occurrence of various clinical features of NF1 vary depending on the feature. In some instances, the effects of unlinked modifying genes may be most important. In other instances, the effects of the normal *NF1* allele may predominate. More than one genetic factor may be involved, and the relative importance of various genetic and non-genetic effects may vary for different features.

Some of the clinical variability that characterizes NF1 may result from allelic heterogeneity of the constitutional *NF1* mutation. Many NF1 patients have been genotyped, but little evidence of allele-phenotype correlation has been observed [Rasmussen and Friedman, 2000]. This may be because phenotypic differences resulting from *NF1* allelic heterogeneity are generally small in comparison to other sources of variability. It is also possible, however, that

important *NF1* genotype-phenotype correlations exist but have not been recognized because of the complexity of the NF1 phenotype [Riccardi, 1999], its strong dependence on age [DeBella *et al.*, 2000b], the non-independence of many clinical features [Szudek *et al.*, 2000b; Szudek *et al.*, (Submitted for publication)], and the heterogeneity of pathogenic *NF1* mutations [Fahsold *et al.*, 2000; Korf, 1999; Messiaen *et al.*, 2000].

Our findings suggest that most NF1 clinical features have important genetic components. The patterns of variable expressivity are subtle, so data will be required on a very large number of patients and/or on very large families to identify modifying genes that affect the NF1 phenotype. Objective quantitative variables such as lesion counts would enable a more detailed analysis of familial segregation patterns and would require fewer patients than binary variables of the type used in the analyses reported here. Given the progressive nature of many NF1 disease features and the potentially confounding effects of age on analysis, it is essential that the data be representative of all age groups. A very dense map of single nucleotide polymorphisms (SNPs) is now available in humans [Sachidanandam *et al.*, 2001], so a random genome scan for NF1 modifying loci is theoretically possible. Improved understanding of neurofibromin's biochemical functions may permit the discovery of interacting proteins and of upstream and downstream effectors that are critical to the development of particular phenotypic features. This would greatly facilitate the identification of modifying loci.

The multivariate probit regression methods used in this study to estimate familial aggregation of NF1 clinical features, while adjusting for age, gender and the presence of other clinical features, are likely to be useful for analysis of other genetic diseases. Application of these methods to mendelian conditions that have highly variable and age-dependent phenotypes,

such as tuberous sclerosis complex [Cheadle *et al.*, 2000], Gorlin syndrome [Wicking and Bale, 1997], and Stickler syndrome [Snead and Yates, 1999] seems especially promising.

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LEGENDS TO FIGURES

Figure 1: Adjusted intrafamilial correlation coefficients and 95% confidence intervals for 6 clinical features among 746 affected first-degree relatives and among 148 affected second-degree relatives. A star indicates a significant difference between the correlation coefficients of the two classes being compared. The multivariate probit regression failed to converge on correlation coefficients between second-degree relatives for optic glioma, other neoplasms, seizures or scoliosis because of the low frequency of these features and insufficient sample size.

Figure 2: Adjusted intrafamilial correlation coefficients and 95% confidence intervals for 6 features among 268 affected sib pairs and among 373 affected parent-child pairs. A star indicates a significant difference between the correlation coefficients of the two classes being compared. The multivariate probit regression failed to converge on correlation coefficients between sibs or parent-child pairs for optic glioma, other neoplasms, seizures or scoliosis.

Figure 3: Adjusted intrafamilial correlation coefficients and 95% confidence intervals for 6 features between 233 affected mother-child pairs and between 140 affected father-child pairs. A star indicates a significant difference between the correlation coefficients of the two classes being compared.

Table I. Number and percentage of subjects from the NFDB and from the study by Easton *et al.* [1993] with various NF1 features. Features not considered by Easton et al. have empty cells in the last two columns.

Feature	NFDB										Easton et al. [1993]	
	Affected Fathers		Affected Mothers		Affected Children of Affected Fathers		Affected Children of Affected Mothers		All Affected Relatives		All Affected Relatives	
	N	%	N	%	N	%	N	%	N	%	N	%
Lisch nodules	63	81%	101	80%	64	63%	101	51%	409	60%		
Café-au-lait macules	67	68%	125	73%	115	79%	181	74%	657	75%	129	88%
Cutaneous neurofibromas	78	80%	119	69%	37	25%	67	27%	355	40%	121	76%
Optic glioma	4	13%	2	4%	8	14%	14	15%	45	15%	9	5%
Subcutaneous neurofibromas	60	61%	99	59%	31	21%	58	23%	291	33%		
Intertriginous freckling	79	83%	147	88%	112	78%	191	78%	699	80%		
Seizures	7	7%	12	7%	8	5%	16	6%	58	6%	12	7%
Plexiform neurofibromas	24	24%	35	20%	25	18%	44	18%	176	20%	37	21%
Scoliosis	7	8%	8	6%	29	22%	32	14%	96	12%	27	16%
Other neoplasms	4	4%	11	6%	4	3%	7	3%	33	4%		

Table II. Summary of regressions in multivariate probit models for 10 clinical NF1 features. The first column lists the 10 modelled features. The second–fourth columns show the covariates and their regression parameter estimates (β) with standard errors (SE) used in each model. β_0 is the intercept in the model equation. Each regression accounts for covariates such as related features, interactions between related features, age and gender. Interactions are depicted by features separated by an "*" and their values equal the product of the two interacting features.

Modelled Feature	Intercept and Covariates	β	SE
Lisch nodules	β_0	0.65	(0.08)
	Age	-3.55	(0.32)
	Male gender	-0.01	(0.08)
	Café-au-lait spots	0.23	(0.15)
	Cutaneous neurofibromas	0.44	(0.20)
	Café-au-lait spots * Cutaneous neurofibromas	-0.09	(0.22)
Café-au-lait spots	β_0	0.28	(0.14)
	Age	-0.66	(0.25)
	Male gender	0.03	(0.09)
	Intertriginous freckling	0.51	(0.12)
	Subcutaneous neurofibromas	-0.41	(0.26)
	Intertriginous freckling* Subcutaneous neurofibromas	0.61	(0.28)
Cutaneous neurofibromas	β_0	-1.62	(0.11)
	Age	-5.56	(0.36)
	Male gender	0.01	(0.10)
	Subcutaneous neurofibromas	0.62	(0.11)
	Plexiform neurofibromas	0.36	(0.12)
Optic glioma	β_0	-1.02	(0.13)
	Age	0.72	(0.57)
	Male gender	0.06	(0.17)
	Plexiform neurofibromas	0.01	(0.37)
	Head circumference	0.19	(0.07)
	Neoplasms	0.55	(0.49)
Subcutaneous neurofibromas	β_0	-1.72	(0.12)
	Age	-3.78	(0.35)
	Male gender	-0.04	(0.08)
	Café-au-lait spots	0.43	(0.11)
	Cutaneous neurofibromas	0.73	(0.13)
	Plexiform neurofibromas	0.52	(0.17)
	Intertriginous freckling * Plexiform neurofibromas	-0.24	(0.23)
Intertriginous freckling	β_0	0.49	(0.15)
	Age	-1.58	(0.30)
	Male gender	-0.23	(0.12)
	Café-au-lait spots	0.52	(0.14)
	Subcutaneous neurofibromas	-0.18	(0.27)
	Lisch nodules	0.55	(0.14)
	Café-au-lait spots * Subcutaneous neurofibromas	0.62	(0.33)

Modelled Feature	Intercept and Covariates	β	SE
Seizures	β_0	-1.43	(0.11)
	Age	-0.88	(0.65)
	Male gender	-0.04	(0.15)
Plexiform neurofibromas	β_0	-1.11	(0.11)
	Age	-0.88	(0.38)
	Male gender	0.07	(0.09)
	Subcutaneous neurofibromas	0.46	(0.16)
	Cutaneous neurofibromas	0.37	(0.14)
	Subcutaneous * Cutaneous neurofibromas	-0.21	(0.22)
Scoliosis	β_0	-1.11	(0.09)
	Age	-0.57	(0.34)
	Male gender	-0.02	(0.11)
Other neoplasms	β_0	-0.95	(0.23)
	Age	-4.07	(2.11)
	Male gender	-0.06	(0.21)
	Lisch nodules	-0.55	(0.25)
	Optic glioma	0.32	(0.31)

Table III. Number of relatives used in multivariate probit models for 10 clinical NF1 features.

Modelled Feature	**Number of Affected Pairs Included for Feature**			
	Sibs	**Mother-Child**	**Father-Child**	**2° Relatives**
Lisch nodules	192	159	79	35
Café-au-lait macules	248	210	129	69
Cutaneous neurofibromas	264	224	131	69
Optic glioma	55	37	26	4
Subcutaneous neurofibromas	253	220	131	69
Intertriginous freckling	179	148	75	35
Seizures	268	233	140	74
Plexiform neurofibromas	264	224	131	69
Scoliosis	228	191	131	53
Other neoplasms	47	33	20	3

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INTRAFAMILIAL CORRELATION OF CLINICAL MANIFESTATIONS IN NEUROFIBROMATOSIS 2 (NF2)

Y. Zhao[1], R.A. Kumar[2], M.E. Baser[3], D.G.R. Evans[4], A. Wallace[4], L. Kluwe[5], V.F. Mautner[6], D.M. Parry[7], G.A. Rouleau[8], H. Joe[2], J.M. Friedman[1]

[1]Department of Statistics and [2]Department of Medical Genetics, University of British Columbia, Vancouver, Canada; [3]Los Angeles, California, USA; [4]Department of Medical Genetics, St. Mary's Hospital, Manchester, UK; [5]Department of Neurology, Klinikum Nord Ochsenzoll, Hamburg, Germany; [6]Department of Neurosurgery, University Hospital Eppendorf, Hamburg, Germany; [7]Genetic Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; [8]Center for Research in Neuroscience, McGill University, Montreal, Quebec, Canada.

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Address correspondence to:

J. M. Friedman, MD, PhD

Department of Medical Genetics

University of British Columbia

6174 University Boulevard – Room 300H

Vancouver, B.C., Canada V6T 1Z3

Telephone: 604-822-0367

E-mail: frid@interchange.ubc.ca

Abstract

Measuring correlation in clinical traits among relatives is important to our understanding of the causes of variable expressivity in mendelian diseases. Random effects models are widely used to estimate intrafamilial correlations, but such models have limitations. We have incorporated survival techniques into a random effects model so that it can be used to estimate intrafamilial correlations in continuous variables with right censoring, such as age at onset. We also describe a negative-binomial gamma mixture model to determine intrafamilial correlations of discrete (e.g., count) data. We demonstrate the utility of these methods by analyzing intrafamilial correlations among patients with neurofibromatosis 2 (NF2), an autosomal dominant disease caused by mutations of the *NF2* tumour suppressor gene.

We estimated intrafamilial correlations in age at first symptom of NF2, age at onset of hearing loss, and number of intracranial meningiomas in 390 NF2 non-probands from 153 unrelated families. A significant intrafamilial correlation was observed for each of the three features: age at onset (0.35; 95% confidence interval [c.i.] 0.23-0.47), age at onset of hearing loss (0.51; 95% c.i. 0.35-0.64) and number of meninginomas (0.29; 95% c.i. 0.15-0.43). Significant correlations were also observed for age at first symptom within NF2 families with truncating mutations (0.41; 95% c.i. 0.06-0.68) or splice-site mutations (0.29; 95% c.i. 0.03-0.51), for age at onset of hearing loss within families with missense mutations (0.67; 95% c.i. 0.18-0.89), and for number of meningiomas within families with splice-site mutations (0.39; 95% c.i. 0.13-0.66). Our findings are consistent with effects of both allelic and non-allelic familial factors on the clinical variability of NF2.

Key words: intrafamilial correlation; random effects model; right censoring; negative-binomial gamma mixture model; neurofibromatosis 2

INTRODUCTION

Variable expressivity is common in mendelian diseases, especially those that are transmitted as autosomal dominant traits. Variable expressivity may be manifested in many different ways, including variation in the age at onset, the types and numbers of clinical features that develop, overall disease severity, rate of progression, length of course, or final outcome. Many different genetic and non-genetic causes of variable expressivity may exist and act alone or in combination [Scriver and Waters, 1999; Dipple and McCabe, 2000].

Random effects models are used to estimate intraclass and intrafamilial associations by dividing phenotypic variance into components that are attributable to different sources of variation. Although methods based on sums of squares are widely used to estimate these variance components, this approach is not applicable when censoring is present. Moreover, since the standard random effects model is based on normality assumptions, it is not appropriate when the data are discrete. In this paper, we have extended the standard random effects model to overcome these limitations. We demonstrate the use of these extended models by analyzing the familiality of selected clinical features of neurofibromatosis 2 (NF2).

NF2 is a highly penetrant mendelian disease that is transmitted as an autosomal dominant trait. The incidence of NF2 at birth has been estimated to be between 1 in 33,000 and 1 in 40,000 [Evans et al., 1992a]. Age at presentation is usually between 11 and 30 years, although younger cases and diagnoses in the fourth and fifth decades also occur [Evans et al., 1992a; Parry et al. 1994]. The hallmark of NF2 is bilateral vestibular schwannomas (VSs), but meningiomas, non-vestibular schwannomas and presenile cataracts are also common. NF2 symptoms are usually related to "tumour burden", i.e., the number, size, and location of tumours, and may include

hearing loss, tinnitus, vertigo, seizures, facial weakness, and visual impairment [Evans et al., 1992c; Parry et al., 1994].

The responsible gene, *NF2,* has been identified and sequenced [Trofatter et al., 1993; Rouleau et al., 1993]. Pathogenic mutations have been found throughout the gene, and a different mutation occurs in almost every family. These mutations are of various types, but most can be classified as nonsense, frameshift, splice-site, missense, or large deletions [MacCollin, 1999].

Clinical studies indicate that the phenotypic expression and natural history of NF2 tend to be similar within a family and that more variability occurs between families [Evans et al., 1992a; Parry et al., 1994, 1996]. Previous studies have demonstrated allele-phenotype correlations for certain *NF2* mutation classes. In general, constitutional truncating mutations (frameshift or nonsense) are associated with severe disease, missense mutations and large deletions with milder disease, and splice-site mutations with variable disease severity, although exceptions do occur [Kluwe et al., 1996, 1998; Parry et al.,1996; Ruttledge et al., 1996; Evans et al., 1998a].

Despite the general similarity in disease severity among affected relatives, substantial phenotypic differences may occur within families [Mautner et al., 1996; Baser et al., 1996b]. It is not known whether this variability occurs by chance or is caused by modifying genes at other loci [Bruder et al., 1999], coincident environmental exposures, or some combination of factors [Baser et al., 1996b].

We have developed statistical methods to estimate the magnitude of intrafamilial correlations for continuous variables with censored observations and for count variables. We have used these methods to test whether the phenotypic similarities found among relatives with NF2 can be explained entirely by the recognized *NF2* mutation class-phenotype correlation. We calculated intrafamilial correlation coefficients (τ) for three clinical features – age at first

symptom, age at onset of hearing loss, and number of intracranial meningiomas – for a large series of NF2 patients and within subgroups of patients with truncating mutations, splice-site mutations, missense mutations, or large deletions of the *NF2* gene. We demonstrate significant intrafamilial correlations for each of these phenotypic features within the entire group of NF2 patients and in one or more subgroups of patients with a particular class of constitutional *NF2* mutations. Our findings suggest that familial factors beyond *NF2* mutation class are important in the pathogenesis of these features in some patients with NF2.

MATERIALS AND METHODS

Statistical Analysis

Random Effects Model for Censored Data

In a random effects model, the total variance for a variable can be separated into two components: variance between families (σ_B^2) and variance within a family (σ_W^2). Let k be the number of families in the study, n_i be the number of affected members in the ith family, and Y_{ij} be the value of the jth patient of the ith family. The statistical model is

$$Y_{ij}=\mu + A_i + \varepsilon_{ij},\ i=1,...k;\ j=1,...n_i$$

where A_i's are independent normal random variables with mean 0 and variance σ_B^2; ε_{ij}'s are also independent random variables with mean 0 and variance σ_W^2. A_i's and ε_{ij}'s are mutually independent. In the above model, μ represents the overall mean of all the individuals; A_i is common to all the members from the same family, representing the deviation of the mean of this particular family from the overall mean μ. The variance of A_i, σ_B^2, reflects the between-family variation, and the variance of ε_{ij}, σ_W^2, reflects the within-family variation. The total variance σ^2 is the sum of σ_B^2 and σ_W^2. When the feature is relatively homogenous within families, σ_W^2 will be

small in comparison to the total variance. Therefore, the strength of intrafamilial resemblance can be measured by the ratio of the between-family variance to the total variance: $\tau = \sigma_B^2 / (\sigma_B^2 + \sigma_w^2)$, i.e. the intrafamilial correlation.

A widely-used procedure for estimating the variance components is to equate sums of squares to their expected values; this approach is not applicable when the variable under consideration is subject to right censoring. Therefore, we used maximum likelihood estimation (MLE) to incorporate survival techniques into a random effects model. Each family in the study contributes one term to the likelihood function. For an individual who has developed the age-dependent feature, we calculate the instantaneous likelihood that the feature occurs at the observed onset age; for an individual who does not have the feature, we calculate the likelihood that the feature occurs beyond the patient's current age. For the ith family, let T_i be the subgroup of all the individuals with the feature and C_i the subgroup of all the individuals without the feature. y_{ij} is the age at onset of the feature if it is present; otherwise, y_{ij} is the patient's age at last examination. The contribution of the family to the likelihood is

$$P_i = f_{T_i}(y_{ij},\ j \in T_i)\ \Pr(Y_{ij'} > y_{ij'},\ j' \in C_i \mid Y_{ij} = y_{ij},\ j \in T_i),$$

where f_{T_i} is the joint density of $\{Y_{ij},\ j \in T_i\}$ and the second term on the right hand side is the conditional probability of $\{Y_{ij'} > y_{ij'},\ j' \in C_i\}$ given $\{Y_{ij} = y_{ij},\ j \in T_i\}$. P_i is parametrized as a function of μ, σ^2 and τ [Jobson, 1996]. The log-likelihood $\Sigma \log(P_i)$ can be maximized numerically with a quasi-Newton method (e.g., Nash 1990) to obtain the maximum likelihood estimates of μ, σ^2 and τ, together with an estimated covariance matrix.

We applied this method to data for two continuous variables available on NF2 patients: age at first symptom and age at onset of hearing loss. For age at first symptom, censoring is present when a patient is asymptomatic at the time of examination or death; for age at onset of hearing

loss, censoring occurs when a patient does not have hearing loss at the time of examination or death.

Random Effects Model for Discrete Data

A random effects model based on a normal distribution is not realistic for a count variable with a high frequency of zeros, such as number of meningiomas in a patient with NF2. We considered using a Poisson distribution to model these data, but the mean and variance are equal in the Poisson distribution. In contrast, the within-family variation is greater than the mean in the NF2 meningioma data. We used a negative-binomial gamma mixture model based on the assumption that the expected count may differ between families as well as within a single family. The similarity within families is represented by a factor with a gamma distribution. For any given family, the count in each member follows a negative-binomial distribution [Lawless, 1987] conditional on the familial factor.

Suppose in the ith family, Y_{ij} is the count in the jth member. We assume that the family factor Λ_i is an unobserved random variable having a gamma distribution with mean 1 and variance $1/\theta$. Conditionally on Λ_i, Y_{ij}'s are independent and have a negative-binomial distribution with mean $\mu_{ij}=\mu_0\Lambda_i$, where μ_0 is the overall mean count across all the families. Given μ_{ij} and another parameter λ, the probability function of the negative-binomial distribution is fully specified as

$$\Pr(Y_{ij} = y) = \frac{\Gamma(\lambda + y)\,\mu_{ij}^{y}\lambda^{\lambda}}{\Gamma(\lambda)y!\,(\mu_{ij}+\lambda)^{\lambda+y}}$$

Since the family factor Λ_i is a random variable, the count per patient varies from family to family. A large variance of Λ_i implies that the families are very different in their means. The correlation between two particular family members, τ, depends on θ, λ and μ_0:

$$\tau = \frac{\mu_0^2 \lambda}{\mu_0 \theta\lambda + \mu_0^2(1+\theta+\lambda)} .$$

The mean μ_{ij} can also be allowed to depend on covariates through a log link function. Let $\boldsymbol{x}_{ij}$ be a vector of covariates and β the vector of coefficients, then $\mu_{ij}=\Lambda_i \exp(\boldsymbol{x}_{ij}\beta)$. The correlation between two particular family members is no longer a constant but instead depends on their $\boldsymbol{x}$-values. If the covariate values of two family members are $\boldsymbol{x}_{ij}$ and $\boldsymbol{x}_{ij'}$, the correlation between them is:

$$\tau = \frac{\mu_j \mu_{j'} \lambda}{\sqrt{[\mu_j \theta\lambda + \mu_j^2(1+\theta+\lambda)][\mu_{j'} \theta\lambda + \mu_{j'}^2(1+\theta+\lambda)]}}$$

where $\mu_k = \exp(\boldsymbol{x}_{ik}\beta)$, $k=j$ or j'.

Note that in the gamma negative-binomial model the variance cannot be partitioned into additive components. Although $1/\theta$ and $1/\lambda$ correspond to the variances of the familial factor and the individual factor, respectively, the total variance is not the sum of these two values. They are not additive because there is additional variation from the Poisson sampling that depends on the mean.

We used this negative-binomial gamma mixture model to assess familiality of meningioma count data in NF2 patients. The maximum likelihood estimates of θ, λ and μ_0, together with an estimated covariance matrix were obtained numerically using a quasi-Newton method [Nash 1990], and the standard error of τ was derived by the delta method [Agresti, 1990]. It would be

appropriate to include covariates such as age, but this information was unavailable for many patients in our data set. Therefore, no covariates were included in the analysis presented below.

Genotype-phenotype Correlations

The constitutional *NF2* mutation was known in a subset of the families, and this permitted us to assess whether the *NF2* allele-phenotype correlation accounts for all of the intrafamilial correlation observed. Patients belonging to families with each of the following four kinds of *NF2* constitutional mutations were analyzed separately: (1) truncating mutations (frameshift or nonsense), (2) splice-site or splice effect mutations, (3) missense mutations, and (4) large deletions.

Intrafamilial correlation coefficients were calculated within subsets of families who shared similar constitutional *NF2* mutation types. To demonstrate the *NF2* genotype-phenotype correlations, we also compared the means of each pair of mutation subgroups simultaneously. The Bonferroni method [Seber, 1977] was used to control the type I error in these multiple comparisons. The z-score was calculated for the difference between each pair of means, but only those with P-value $< \alpha/k$ were considered to be statistically significant, where α was chosen as 0.05 and k is the total number of pairs tested (6 in this instance).

Patients

390 patients from 153 families were ascertained from both published and unpublished sources (Supplemental Table). All patients included met the Manchester clinical diagnostic criteria for NF2 (Evans et al., 1992b), had an identified constitutional *NF2* mutation, or both. Probands were excluded from the table and from all statistical analyses to avoid ascertainment

bias. All other affected individuals were included if clinical information was available for at least one of the three manifestations studied: age at first symptom, age at onset of hearing loss, or number of intracranial meningiomas. These variables were examined because they were the most reliably reported features across the various data sources used for the study. Meningiomas were identified by cranial CT or MRI scan. Only intracranial meningiomas were considered in this study. The total numbers of families and patients used to examine each clinical feature are given in Table 1.

Age at first symptom of NF2 and age at onset of hearing loss are both subject to right censoring. Censoring can occur either because the manifestation was not present at the time of last evaluation or because the manifestation was not present when the subject died. Death accounts for a small proportion of censored cases in this data set.

RESULTS

Among the 390 NF2 patients included in this study, 300 (76.9%) had bilateral VSs, 31 (7.9%) had unilateral VS, 26 (6.7%) had no VS, and in 33 cases (8.5%) the VS status was unknown.

Age at First Symptom

373 patients from 150 families were included in the study of age at first symptom. 72 (19%) of the patients were asymptomatic at the time of last examination or death and were, therefore, treated as right-censored cases in this analysis. Among the symptomatic patients, age at first symptom ranged from 1 to 62 years.

To assess the assumption of normality for age at first symptom, we examined normal probability plots for all subjects together and for subjects in each mutation subclass. These plots

did not show extreme skewness, except in the subclass of patients with large deletion mutations, where the distribution was skewed to the right. The random effects model was also fit in this subgroup using log-transformed age at first symptom. The estimate of τ was about the same, so only the results of the model using untransformed values of age are reported here.

Table 2 shows the mean, standard deviation, and intrafamilial correlations calculated for affected members of all families included in this study as well as for members of families with each of four types of constitutional *NF2* mutations: truncating mutations, splice-site mutations, missense mutations and large deletions. The value of τ within each subgroup of mutations except large deletions was similar in magnitude to that seen when all families were analyzed together. For all *NF2* mutations considered together, the intrafamilial correlation coefficient for age at first symptom was 0.35, and the lower 95% confidence limit was 0.23. The 95% confidence intervals for τ were always wider in the subgroups, as expected with smaller sample sizes. Nevertheless, in two of the subgroups (truncating mutations and splice-site mutations), the lower limit of the 95% confidence interval of τ excluded 0.

We conducted pairwise tests to assess differences between the mean ages at first symptom in the subgroups and tested the nominal statistical significance using the Bonferroni method. The mean age at first symptom in the subgroup with truncating mutations was significantly different from the mean age at first symptom in the splice-site and missense subgroups, whereas the differences between all the other pairs were not statistically significant. Patients with truncating mutations had an earlier mean age at first symptom (18.7 years) and less variation (standard deviation, 9.5 years). The pattern of age at first symptom is shown more clearly in the Kaplan-Meier estimates of the proportion of asymptomatic patients at various ages for each mutation type (Figure 1).

Age at Onset of Hearing Loss

Of 261 NF2 patients from 114 families for whom hearing status was known, 192 individuals (74%) had lost their hearing at the time of examination. The age at onset of hearing loss among these patients ranged from 3 to 62 years. 69 (26%) of the 261 patients did not have hearing loss at the time of last examination or death and were treated as right-censored in the analysis.

To assess the assumption of normality for age at onset of hearing loss, we examined normal probability plots for all patients together and for each mutation subclass. The distribution for all cases together was not skewed, but right skewness was observed for all subclasses except truncating mutations. A logarithmic transformation provided a better fit for the subgroups that had a skewed distribution, but the estimates of τ remained almost the same as without the transformation. For this reason, we only report results for the analysis without transformation of age.

The means, standard deviations and intrafamilial correlations for age at onset of hearing loss are reported in Table 3, and the Kaplan-Meier estimates are plotted in Figure 2. A strong intrafamilial correlation was seen for age at hearing loss when all patients were considered together ($\tau = 0.51$, 95% c.i. 0.35-0.64). Within the subgroups defined by constitutional *NF2* mutation type, those with missense mutations had a somewhat higher intrafamilial correlation than the other subgroups, and it was only in this subgroup that the 95% confidence interval of the correlation coefficient excluded zero.

Pairwise tests showed that the mean age at onset of hearing loss for patients with truncating mutations was significantly lower than that of patients with splice-site or missense mutations. The means of the other subgroups did not differ significantly from each other.

Number of Intracranial Meningiomas

259 NF2 patients from 122 families were used in the study of intracranial meningiomas. The distribution of number of meningiomas is summarized in Table 4 by mutation type, and estimates of the model parameters and intrafamilial correlations are reported in Table 5.

The mean number of intracranial meningiomas per patient was 1.01 (95% c.i. 0.70-1.32). A significant intrafamilial correlation for number of meningiomas was observed for all NF2 patients combined ($\tau = 0.29$, 95% c.i. 0.15-0.43).

NF2 patients with truncating mutations had the highest mean number of meningiomas, 1.92 (95% c.i. 1.02-2.82), but this was associated with relatively high within-family variance. The magnitude of the intrafamilial correlation coefficient was small in this subgroup.

The mean number of intracranial meningiomas among NF2 patients with splice-site mutations was 0.72 (95% c.i. 0.29-1.15). In contrast to the situation with truncating mutations, the within-family variation was small and the between-family variation was large for NF2 patients with splice-site mutations. The point estimate of the intrafamilial correlation coefficient was higher in this subgroup than in any of the other mutation subgroups, and the 95% confidence interval excluded zero.

The mean number of intracranial meningiomas among NF2 patients with missense mutations was 0.42 (95% c.i. 0.09-0.75), the lowest among the four mutation subgroups because 16 of the 23 patients in this subgroup had no meningiomas. The variation both between families

and within a family was similar in magnitude to the mean, and the intrafamilial correlation coefficient was small.

The mean number of intracrainal meningiomas among NF2 patients with large deletions was 0.96 (95% c.i. 0-2.02). The within- and between- family variances were both large, mainly because of one patient (#201 in family 1648) who developed 19 meningiomas by age 18 (Bruder, 2001). More than 2/3 of individuals with this mutation type had no meningiomas, and all of the others had either one or two meningiomas, including patient 201's two affected relatives. When this family was excluded from the analysis, the mean number of meningiomas among the remaining patients with large deletions was 0.39, both within- and between-family variation were much smaller, and the intrafamilial correlation was even lower (0.06).

DISCUSSION

Statistical Methods

The statistical methods used in this paper should be of use in intrafamilial correlation studies of other genetic diseases. Random effects models are commonly used to analyze intraclass and intrafamilial correlations in continuous traits, and we have extended this method to include right-censored data. The maximum likelihood method we describe can also accommodate two other types of censoring frequently associated with age-related traits: left censoring (e.g., the event occurred before the time of examination) and interval censoring (e.g., the event occurred between two examinations). A mixed-effects model can be used to adjust the correlations calculated by this method for covariates [Searle, 1992].

The negative-binomial gamma mixture model we developed for count traits is also likely to be useful for other genetic diseases. A Poisson mixture model is sometimes used with count data [Foulley et al., 1987], but the Poisson distribution is constrained because the variance is equal to

the mean. A mixture model based on the negative-binomial distribution allows more flexibility and is, therefore, more appropriate for count variables with overdispersion relative to the Poisson distribution [Tempelman, 1996].

Intrafamilial Correlations in NF2

Phenotypic variability is observed in individuals with NF2, both within and between families. We employed a random effects model incorporating survival techniques to estimate intrafamilial correlations in two continuous variables that are right-censored – age at first symptom and age at onset of hearing loss. We used a negative-binomial gamma mixture model to estimate intrafamilial correlations for a discrete variable – number of intracranial meningiomas. Our results demonstrate that relatives with NF2 are more similar to each other than to unrelated affected individuals with respect to each of these clinical features. These observations are consistent with anecdotal clinical experience [Evans et al., 2000]. Parry et al. [1996] adjusted for intrafamilial correlation in their genotype-phenotype analysis, but the intrafamilial correlation of NF2 phenotypes has not previously been tested statistically.

Intrafamilial correlations, such as those observed in this study, may have a variety of causes. Effects of the mutant allele, of other shared genes, of shared environmental factors, or of a combination of genetic and environmental factors may produce such correlations. To distinguish between these possibilities requires analysis of phenotypic correlations among affected family members of various classes, such as monozygotic twins, sibs, parent-child pairs, and more distant relatives.

Since all affected individuals in the same family can be presumed to carry the same constitutional alteration of the *NF2* locus, the nature of the *NF2* mutation itself might account for the familiality we observed. This possibility is supported by the associations observed in cross-

sectional studies between allele class and disease severity in NF2 [Kluwe et al., 1996, 1998; Parry et al., 1996; Ruttledge et al., 1996; Evans et al., 1998a]. Our study includes data on patients who are also included in these earlier studies, and, as expected, we found similar effects.

We also observed intrafamilial correlations of similar or greater magnitude for each of the features studied in subgroups of patients who all had the same type of constitutional *NF2* mutation. While it is possible that specific allelic differences within each mutation class account for these intrafamilial correlations, our findings could also reflect the effects of modifying genes. Recent reports of putative modifying loci for *NF2* are consistent with this interpretation [Bruder et al., 1999; Goutebroze et al., 2000]. Several genes other than *NF2* have been implicated in meningioma development, including loci on chromosomes 1, 3, 6, 7 and 22 [Sanson et al., 1993; Sulman et al., 1998; Comtesse et al., 1999], but the contribution of these loci to the interfamilial variability observed in NF2 pedigrees is unknown.

Our studies are subject to several limitations. We used data from a variety of sources, and differences in referral patterns, diagnostic acumen, and criteria for diagnosis probably exist among the centers. Age at first symptom and age at onset of hearing loss are taken from published data (including updated data provided by the authors) and unpublished data. The definitions of these ages may vary from source to source. In many cases, the age at first symptom and age at onset of hearing loss were assigned retrospectively and thus may be subject to recall errors. All of these factors could affect the accuracy of our results.

Although this study was based on the largest collection of clinical data available on NF2 patients, consideration of the separate mutation types was limited by small sample sizes. Consequently, our estimates of τ for the subgroups are associated with wide confidence intervals. Some of the correlations that did not appear to be significant in this study might be important but

require larger samples for demonstration. A likelihood ratio test could be performed to assess whether the intrafamilial correlations vary significantly with mutation type. This would be useful in a study that includes more patients, but a likelihood ratio test would not show significant differences because of the small sample size of each subgroup in the present study.

The penetrance of NF2 and the prevalence of individual tumor types generally increase with age [Mautner et al., 1993; MacCollin and Mautner, 1998]. Time from onset of symptoms may also influence the number of meningiomas in an NF2 patient, so it would be appropriate to model this time variable as an additional source of variation that is independent of the familial factor. Unfortunately, we did not know the age at which meningioma status was determined for many of the patients in this study, so we could not include age as a covariate in our analysis.

Statistical techniques provide powerful means of studying genetic and non-genetic aspects of diseases such as NF2. Methods are needed to estimate intrafamilial correlations for other kinds of non-normally distributed traits, such as ordered categorical data (e.g., severity of disease) and continuous data that are not normally distributed (e.g., disease progression rate). Each of these data types requires a different statistical model to capture the specific distributional features. Models that allow a wide range of dependent structures, so that various genetic and environmental components of phenotypic variation can be assessed at the same time, are especially desirable.

Table 1. Number of NF2 families and patients included for each of the clinical features examined.

Mutation Type and Clinical Feature	Number of Families	Number of Patients
All Mutation Types		
Age at first symptom	150	373
Age at onset of hearing loss	114	261
Number of intracranial meningiomas	122	260
Truncating (nonsense or frameshift)		
Age at first symptom	37	58
Age at onset of hearing loss	25	39
Number of intracranial meningiomas	30	44
Splice-site		
Age at first symptom	32	101
Age at onset of hearing loss	23	60
Number of intracranial meningiomas	27	79
Missense		
Age at first symptom	12	50
Age at onset of hearing loss	9	38
Number of intracranial meningiomas	10	23
Large Deletions		
Age at first symptom	13	42
Age at onset of hearing loss	11	34
Number of intracranial meningiomas	12	36

Table 2. Mean, standard deviation and intrafamilial correlation of the age at first symptom in 373 NF2 patients from 150 families. Approximate 95% confidence intervals of the point estimates are given in brackets.

Mutation Type	Censoring Rate	Mean	Standard Deviation	Intrafamilial Correlation (τ)
All mutation types	19%	24.9 (23.1, 26.8)	13.1 (12.0,14.2)	0.35 (0.23, 0.47)
Truncating (nonsense or frameshift)	15%	18.7 (15.6, 21.9)	9.5 (7.6, 11.5)	0.41 (0.06, 0.68)
Splice-site	19%	25.1 (21.6, 28.5)	12.1 (10.0, 14.2)	0.29 (0.03, 0.51)
Missense	20%	29.3 (24.1, 34.6)	11.9 (8.9, 14.9)	0.32 (0, 0.61)
Large deletions	14%	24.5 (20.0, 29.0)	11.3 (8.7, 14.0)	0.10 (0, 0.34)

Table 3. Mean, standard deviation and intrafamilial correlation of the age at onset of hearing loss for 261 NF2 patients from 114 families. Approximate 95% confidence intervals of the point estimates are given in brackets.

Mutation Type	Censoring Rate	Mean	Standard Deviation	Intrafamilial Correlation (τ)
All mutation types	26%	29.6 (27.2, 32.1)	13.3 (11.8,14.9)	0.51 (0.35, 0.64)
Truncating (nonsense or frameshift)	23%	22.2 (19.3, 25.1)	7.4 (5.5, 9.4)	0.41 (0, 0.76)
Splice-site	40%	31.6 (27.4, 35.9)	11.7 (8.9, 14.5)	0.29 (0, 0.62)
Missense	24%	36.9 (27.4, 46.4)	14.9 (8.1, 21.7)	0.67 (0.18, 0.89)
Large deletions	26%	28.9 (22.2, 35.6)	13.0 (9.2, 16.8)	0.19 (0, 0.55)

Table 4: Distribution of number of meningiomas in 259 NF2 patients from 122 families.

Mutation	Frequency of number of meningiomas										
Type	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**9**	**10**	**19**
All mutation types	164	50	19	8	6	5	2	1	2	1	1
	63.3%	19.3%	7.3%	3.1%	2.3%	1.9%	0.8%	0.4%	0.8%	0.4%	0.4%
Truncating*	18	9	4	4	4	1	1	1	1	1	0
	40.9%	20.5%	9.1%	9.1%	9.1%	2.3%	2.3%	2.3%	2.3%	2.3%	
Splice-site	50	19	6	1	1	1	0	0	1	0	0
	63.3%	24.1%	7.6%	1.3%	1.3%	1.3%			1.3%		
Missense	16	6	0	1	0	0	0	0	0	0	0
	70.0%	26.1%		4.3%							
Large deletions	25	4	6	0	0	0	0	0	0	0	1
	69.4%	11.1%	16.7%								2.8%

* nonsense and frameshift

Table 5. Parameter estimates and intrafamilial correlation coefficients for number of meningiomas. Approximate 95% confidence intervals for the point estimates are given in brackets.

Mutation Type	$1/\theta$*	$1/\lambda$**	Mean (μ_0)	Intrafamilial Correlation (τ)
All mutation types	1.24	0.93	1.01	0.29
	(0.53, 1.95)	(0.32, 1.54)	(0.70, 1.32)	(0.15, 0.43)
Truncating (nonsense and frameshift)	0.29	1.19	1.92	0.12
	(0.00, 0.58)	(0.17, 2.21)	(1.02, 2.82)	(0.00, 0.25)
Splice-site	1.43	0.34	0.72	0.39
	(0.14, 2.72)	(0, 0.89)	(0.29, 1.15)	(0.13, 0.66)
Missense	0.28	0.47	0.42	0.08
	(0, 0.69)	(0, 2.45)	(0.09, 0.75)	(0, 0.23)
Large deletions	1.25	2.37	0.96	0.16
	(0, 3.94)	(0, 5.96)	(0, 2.02)	(0, 0.45)

*$1/\theta$: variance between families

**$1/\lambda$: variance within a family

FIGURE LEGENDS

Fig. 1. Kaplan-Meier estimates of probability of remaining asymptomatic at a given age.

Fig. 2. Kaplan-Meier estimates of probability of NOT developing hearing loss by a given age.

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Supplemental Table. NF2 families and patients included in the study.

Type of mutation and references	Family Number	Relatives Included	Mutation
Nonsense			
[a]Evans et al.	165	1	E2: C586→T
	1556	1	E15: C1606→T
	1557	2	E2: C169→T
	1614	2	E2: G122→A
	1615	3	E14: G1570→T
	1894	1	E8: C784→T
	2267	1	E2: C169→T
	2286	2	E13: C1408→T
[b]Ruttledge et al.	204	1	E15:1580 GAA (Glu) →TAA (Stop)
	209	1	E11:1021 CGA (Arg) →TGA (Stop)
	540	1	E11:1021 CGA (Arg) →TGA (Stop)
	716	2	E11:1021 CGA (Arg) →TGA (Stop)
[c]Parry et al.	FF5863	1	E3: 331 C (Gln)→T (Stop)
	G1703	2	E11: 1021C (Arg) →T (Stop)
	G17690	2	E2: 169 C (Arg) →T (Stop)
	G17693	2	E13: 1396 C(Arg) →T (Stop)
	G17696	1	E15: 1606 C(Gln) →T (Stop)

	G17900	4	E11: 1021 C(Arg) →T (Stop)
[d]Kluwe et al.	32	1	E8:784C (Arg) →T (Stop)
[e]Mérel et al.	GL2	1	E11: C→T
	IC12	1	E11: C→T
Frameshift			
[a]Evans et al.	139	1	E1: 40-1 del CT
	160	1	E8: 713 del C
	1560	2	E8: 678 ins C
	1600	1	E1: 36 del CTCT
	1665	1	E1: 40-1 del CT
	1671	1	E2: 183 ins CAAA
	1672	3	E9: 855 del T
	1675	2	E10: 953 del T
	2271	1	E6: 523-4 del AA
[b]Ruttledge et al.	260	4	E14: 1564 –1567 del GAGA
	725	1	E14: 1518 –1520 ins T
[c]Parry et al.	G6272	2	E14: 1499 del T
[d]Kluwe et al.	86	2	E8: 761 ins TC
[e]Mérel et al.	GL18	1	E8: del C
	RE1	1	E11: GAACGCACGAGG → CGAGAGAAGCA
[f]Kluwe et al.	KM10	2	1562 - 1564 ins A
Splice donor site			

[a]Evans et al.	1613	1	E5: 516+1 G→A
	1660	2	E6: 599+1 G→A
	1664	4	E2: 240+1 G→T
[b]Ruttledge et al.	222	1	E11: CTG: gtg → CTG: ttg
	234	5	E8: GAG: gta → GAA: gta
[c]Parry et al.	G17302	6	E8: 810+ 2 ins 3bp
	G17695	2	E5: 516+lg → a
[e]Mérel et al.	GL5	1	E:12: 1737AG:gtAG:at
	GL9	1	E2: AG:gt→AG:tt
[g]Kluwe et al.	12	3	E15: AG:gt→AT:gt
	139	1	E14: AA:gt→AA:at
[h]Bijlsma et al.	EBD	4	E5: 516 AGG: gt→AGG: at
[i]Sainio et al.	1	10	E15: 1737+3 A→T
Splice acceptor site			
[a]Evans et al.	156	3	E7: 600-3 C→G
	170	2	E14: 1447-1 G→A
	1505	8	E5: 448-1 G→T
	1555	3	E5: 448-1 G→A
	1596	2	E7: 602-2 A→G
	2275	3	E8: 676-7 T→G
	2298	1	E3: 241-9 A→G
	2373	2	E10: 886-1 G→A

[b]Ruttledge et al.	213	1	E13: cag: GGC→caa: GGC
	779/21	2	E15: cag: AGT→cac: AGT
[c]Parry et al.	G16209	2	E5: 448-2 a→g
	G17697	2	E13: 1341-1g→a
[e]Kluwe et al.	20	1	E5: 481-1ag: TA→at: TA
	82	6	E14: 1447-2ag: CC→gg: CC
	118	3	E15: ag: Ag→aa: AG
[j]Hung et al.	81	1	E15:1575-1 G→A
[k]Zucman-Rossi et al.	2(GL20)	1	E14: cag: CC→cg: CC
Splicing error			
[b]Ruttledge et al.	203	3	E14: AG: gtacccaggg ins 200bp
[l]Wertelecki et al.	1	23	E7: A (Tyr) →T (Asn) *
[m]De Klein et al.	1	3	E5: 301 GA
Missense			
[a]Evans et al.	146	2	E2: A229G→A
	1647	3	E2: T185→C
	1651	3	E7: T623→G
	1659	10	E15: T1604→C
	1697	1	E12: C1055→T
	2395	3	T1193→C
[b]Ruttledge et al.	256	5	E11: 1079 CTG (Leu) →CCG (Pro)
[d]Kluwe et al.	70	1	E11: 1079 CTG (Leu) →CCG (Pro)

	14	2	E15: 1613 A (Gln) →C (Pro)
[e]Mérel et al.	RF10	1	E11: T→C
[n]Baser et al.	1	16	
[o]Scoles et al.	1	3	E2: 185 T (Phe) →C (Ser)
In-frame	**deletion/insertion**		
[b]Ruttledge et al..	736	10	E2: 49 ins GAT TTG→ GAT TTG TTG
[c]Parry et al.	G5772	11	E13-14: del 1341 - 1574
Large deletion			
[a]Evans et al.	29	5	Whole gene
	1648	3	Whole gene
	1653	9	Whole gene
	1662	1	Probably whole gene
	1668	2	Whole gene
	1677	1	E9-15: 60 kb
	1687	4	100kb around intron 2
	2269	2	Part gene
	2276	1	Part gene
	2280	8	Whole gene
	2284	3	Whole gene
[k]Zucman-Rossi et al.	3	3	E1-17
[p]López-Correa et al.	1	1	At least intron 1-intron 10
Chromosomal	**Rearrangement**		
[a]Evans et al.	2362	1	

Unidentified

[a]Evans et al.	149	1
	154	2
	1519	4
	1572	4
	1592	1
	1597	1
	1601	5
	1633	1
	1644	1
	1661	1
	1674	1
	1696	1
	1891	1
	2265	1
	2268	3
	2270	1
	2367	1
	2389	1
	2390	1
[b]Ruttledge et al.	1	6
	5	2
	10	2

	11	2
	16	5
	19	4
	20	1
	21	1
	23	2
	25	1
	40	1
	41	1
	47	1
	53	1
	56	1
	63	1
	68	1
	69	1
[c]Parry et al.	FF5734	1
	G17709	3
	G17710	1
	G17901	1
	G19758	4
	G19759	1
[j]Hung et al.	79	1
	139	1

	190	1
[k]Zucman-Rossi	5	1
et al.	6	1
	7	1
[n]Baser et al.	2	7
	5	8
[q]Baser et al.	1	2
	2	1
	3	1

*Based on the base pair alteration on the exon, the mutation of this family is classified as a missense mutation although it appears to function as a splicing error (Jacoby et al., 1999).

[a]Evans et al., 1998a; Evans et al., 1998b; D. G. R. Evans, unpublished data

[b]Ruttledge et al., 1996; G. A. Rouleau, unpublished data

[c]Parry et al., 1996; D. M. Parry, unpublished data

[d]Kluwe et al., 1996

[e]Merel et al., 1995

[f]Kluwe et al., 2000

[g]Kluwe et al., 1998

[h]Bijlsma et al., 1995

[i]Sainio et al., 1995 and 2000

[j]Hung et al., 2000

[k]Zucman-Rossi et al., 1998

[l]Wertelecki et al., 1988; MacCollin et al., 1993; Jacoby et al., 1999 (E7); D. M. Parry, personal communication

[m]De Klein et al., 1998

[n]Baser et al., 1996a

[o]Scoles et al., 1996

[p]Lopez-Correa et al., 2000

[q]Baser et al., 1996b

Baser ME, Friedman JM, Joe H, Wallace AJ, Ramsden RT, Evans DGR. **Genotype-phenotype correlations for presenile cataracts in neurofibromatosis 2.** American Society of Human Genetics 52nd Annual Meeting, 2002 October 15-19, Baltimore (MD). Accepted.

Neurofibromatosis 2 (NF2) is an autosomal dominant disease that is characterized by nervous system tumors and other abnormalities. Genotype-phenotype correlations have been studied for NF2-related tumors, but have not been evaluated for non-tumor manifestations of NF2, the most common of which is presenile cataracts. We used data from the United Kingdom NF2 registry to examine genotype-phenotype correlations for cataracts in 261 people from 191 families (160 new mutations and 101 inherited cases) that had been screened for constitutional *NF2* mutations using SSCP. Cataracts were evaluated using slit lamp examinations. There were 82 people with nonsense or frameshift mutations (including 15 somatic mosaics), 50 with splice-site mutations, 16 with missense mutations, 32 with large deletions, and 83 with unidentified mutations. Logistic regression was used to calculate relative risks (RR). The RR of cataracts was not significantly associated with age at diagnosis of NF2 or current age. People with nonsense or frameshift mutations were the reference group in comparisons between types of mutations. In new mutations, the RR of cataracts in people with splice-site mutations was 0.94 (95% confidence interval (CI), 0.31-2.80); with missense mutations, 0.96 (95% CI, 0.14-6.47); with large deletions, 0.39 (95% CI, 0.07-2.14); with somatic mosaicism, 0.29 (0.07-1.17); and with unidentified mutations, 0.30 (95% CI, 0.13-0.68). In inherited cases, the RR of cataracts in people with splice-site mutations was 0.42 (95% CI, 0.13-1.29); with missense mutations, 0.18 (95% CI, 0.03-1.06); with large deletions, 0.60 (95% CI, 0.18-1.91); and with unidentified mutations, 0.46 (95% CI, 0.12-1.84). In future work, we will evaluate age at diagnosis of cataract, which may be a more sensitive measure than presence of cataract.

Tzenova J, Joe H, Riccardi VM, Friedman JM. **The effect of parental age on the occurrence of neurofibromatosis 1.** *Am J Hum Genet 69 (Suppl):393, 2001.*

New mutations account for half of all patients with neurofibromatosis 1 (NF1), and about 80% of new mutations occur in the paternally-inherited allele. The exception is large deletions, which are predominantly maternal in origin. Typical NF1 may also occasionally result from somatic mutations. Previous studies of paternal age among patients with sporadic NF1 have been inconclusive. We postulated that failure to find a paternal age effect in these studies may have resulted from inclusion of patients with large deletions and somatic mutations, for whom no paternal age effect would be expected.

In order to test this possibility, we used data collected from 280 sporadic and 389 familial NF1 patients. We excluded 11 (3.9%) exceptionally mild sporadic cases as possible somatic mosaics and 14 (5%) sporadic cases with the large deletion phenotype. We compared paternal age in the remaining 255 sporadic probands to 100 familial probands for whom both the paternal and maternal ages at birth were known. The mean paternal age for the sporadic probands (31.71 years) was significantly greater ($p=.017$) that that for familial probands (29.87 years). The results were unaffected by ethnicity or year of ascertainment.

We also studied the effect of maternal age on transmission of the abnormal NF1 allele. We found no significant difference between the maternal age at birth of 74 proband children of affected mothers and that of the 63 proband children of affected fathers. Logistic regression of affection status in 334 children of affected women and 146 children of affected men in 239 families showed no significant relationship with gender of the affected parent, maternal age at birth of the child, paternal age at birth of the child, or birth order. Probands were excluded from this analysis to reduce ascertainment bias. We conclude that there is suggestive evidence for a paternal age effect on the occurrence of sporadic NF1 but that neither maternal nor paternal age at birth affects the occurrence of familial NF1.

Zhao Y, Kumar RA, Baser ME, Evans DGR, Wallace A, Rouleau GA, Mautner VF, Kluwe L, Joe H, Friedman JM. **Allele class-independent intrafamilial correlation of age at onset, age at hearing loss and number of intracranial meningiomas in neurofibromatosis 2 (NF2).** *Am J Hum Genet;*69(4 Suppl):255, 2001.

Mutation of the NF2 tumour suppressor gene causes NF2, an autosomal dominant disease characterized by very high frequencies of vestibular schwannomas, schwannomas of other nerves, meningiomas, and gliomas. The disease is highly penetrant but manifests variable expressivity both within and between families. We have developed statistical models to quantify familial correlations of age at onset of the presenting symptom, age at onset of hearing loss, and number of intracranial meningiomas. A random effects model employing survival techniques to account for right censoring was used for age at onset of the presenting symptom and age at hearing loss. A negative binomial gamma mixture model with penetrance dependent on time since onset was used for number of meningiomas.

Using these models, we demonstrated a significant intrafamilial correlation for each of the three features in 377 NF2 non-probands from 154 unrelated families: age at onset (tau=.06; 95% confidence interval 0.23-0.47), age at hearing loss (0.40; 0.23-0.55) and number of meningiomas (0.20; 0.04-0.36). Significant correlations were also observed within NF2 families with various specific classes of mutations: truncating mutations – age at first symptom (0.41;0.05-0.67) and number of meningiomas (0.09; 0.01-0.17); splice site mutations – age at first symptom (0.28; 0.04-0.49) and number of meningiomas (0.37;0.13-0.61); and missense mutations – age at hearing loss (0.64; 0.16-0.88). Our findings are consistent with effects of both allelic and non-allelic familial factors on the clinical variability of NF2.